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TABLE OF CONTENTS

Guidelines	Index	Most Recent Date of Issue
I. GENERAL TOXICITY TESTING		
Acute Exposure		
Dermal Toxicity	HG-Acute-Dermal	1982
Inhalation toxicity	HG-Acute-Inhal	1983
Oral Toxicity	HG-Acute-Oral	1983
Subchronic Exposure		
Dermal Toxicity	HG-Subchronic-Dermal	1983
Inhalation Toxicity	HG-Subchronic-Inhal	1983
Oral Toxicity	HG-Subchronic-Oral	1983
Chronic Exposure		
Chronic Toxicity	HG-Chronic	1983
Oncogenicity	HG-Chronic-Onco	1983
Combined Chronic Toxicity/ Oncogenicity	HG-Chronic-Combined	1983
II. SPECIFIC ORGAN/TISSUE TOXICITY		
Dermal Sensitization	HG-Organ/Tissue-Dermal Sensit	1982
Primary Dermal Irritation	HG-Organ/Tissue-Dermal Irrit	1983
Primary Eye Irritation	HG-Organ/Tissue-Eye Irrit	1982
Reproduction/Fertility Effects	HG-Organ/Tissue-Repro/Fert	1983
Teratogenicity	HG-Organ/Tissue-Terato	1983
III. MUTAGENICITY		
Gene Mutations		
Salmonella typhimurium	HG-Gene Muta-*S. typhimurium*	1983
Escheria coli WP2 and and WP2 *uvrA*	HG-Gene Muta-*E. coli*	1983
Aspergillus nidulans	HG-Gene Muta-*A. nidulans*	1983
Neurospora crassa	HG-Gene Muta-*N. crassa*	1983
Sex Linked Recessive Lethal Test in *Drosophila melanogaster*	HG-Gene Muta-Insects	1983
Somatic Cells in Culture	HG-Gene Muta-Somatic Cells	1983
Mouse Specific-Locus Test	HG-Gene Muta-Mammal	1983

Guidelines	Index	Most Recen Date of Issue
Chromosomal Effects		
In Vitro Mammalian Cytogenetics	HG-Chromo-In vitro	1983
In Vivo Mammalian Bone Marrow Cytogenetics Tests Chromosomal Analysis	HG-Chromo-Bone Marrow	1983
In Vitro Sister Chromatid Exchange Assay	HG-Chromo-Sister-Chrom-In vitro	1983
In Vivo Sister Chromatid Exchange Assay	HG-Chromo-Sister-Chrom-In vivo	1983
In Vivo Micronucleus Assay	HG-Chromo-Micronuc	1983
Heritable Translocation Test in *Drosophila melanogaster*	HG-Chromo-Insects	1983
Dominant Lethal Assay	HG-Chromo-Dom Lethal	1983
Rodent Heritable Translocation Assay	HG-Chromo-Herit Translocat	1983
DNA Effects		
Differential Growth Inhibition of Repair Deficient Bacteria: "Bacterial DNA Damage or Repair Tests"	HG-DNA-Damage/Repair	1983
Unscheduled DNA Synthesis in Mammalian Cells in Culture	HG-DNA-Unsched Syn	1983
Mitotic Gene Conversion in *Saccharomyces cerevisiae*	HG-DNA-Gene Conversion	1983
IV. NEUROTOXICITY		
Neuropathology	HG-Neuro-Path	1983
Support Document	HS-Neuro-Path	1983
Peripheral Nerve Function	HG-Neuro-Peri Nerve	1983
Support Document	HS-Neuro-Peri Nerve	1983
Motor Activity	HG-Neuro-Motor Act	1983
Support Document	HS-Neuro-Motor Act	1983
Acute Delayed Neurotoxicity of Organophosphorus Substances	HG-Neuro-Acute Delayed	1983
Subchronic Delayed Neurotoxicity of Organophosphorus Substances	HG-Neuro-Subchronic Delayed	1983
Functional Observational Battery	HG-Neuro-Battery	1983
Support Document	HS-Neuro-Battery	1983
Schedule-Controlled Operant Behavior	HG-Neuro-Operant Behavior	1983
Support Document	HS-Neuro-Operant Behavior	1983

Guidelines	Index	Most Recent Date of Issue
V. SPECIAL STUDIES		
Metabolism	HG-Spec Stud-Metab	1983

HG-Acute-Inhal
1983

ACUTE EXPOSURE
INHALATION TOXICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

In the assessment and evaluation of the toxic characteristics of an inhalable material, such as a gas, volatile substance or aerosol/particulate, determination of acute inhalation toxicity is usually an initial step. It provides information on health hazards likely to arise from short term exposure by the inhalation route. The purpose of an acute inhalation study is to determine the median lethal dose (LC50), its statistical limits and slope using a single exposure, usually of 4 hours, and a 14-day post-exposure observation period. This purpose can be accomplished by performing the provisions contained in this guideline. Data from an acute study serves as a basis for classification and labelling. It is also an initial step in establishing a dosage regimen in subchronic and other studies. With the addition of certain other test elements, this guideline may provide information on the mode of toxic action of a substance.

II. DEFINITIONS

A. Acute inhalation toxicity is the adverse effects caused by a substance following a single uninterrupted exposure by inhalation over a short period of time (24 hours or less) to a substance capable of being inhaled.

B. Aerodynamic diameter applies to the size of particles of aerosols. It is the diameter of a sphere of unit density which behaves aerodynamically as the particle of the test substance. It is used to compare particles of different size and densities and to predict where in the respiratory tract such particles may be deposited. This term is used in contrast to measured or geometric diameter which is representative of actual diameters which in themselves cannot be related to deposition within the respiratory tract.

C. The geometric mean diameter or the median diameter is the calculated aerodynamic diameter which divides the particles of an aerosol in half based on the weight of the particles. Fifty percent of the particles by weight will be larger than the median diameter and 50 percent of the particles will be smaller than the median diameter. The median diameter and its geometric standard deviation is used to statistically describe the particle size distribution of any aerosol based on the weight and size of the particles.

D. Inhalable diameter refers to that aerodynamic diameter of a particle which is considered to be inhalable for the organism. It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract from the trachea to the alveoli. For man, the inhalable diameter is considered as 15 micrometers or less.

E. The LC50 (median lethal concentration) is a statistically derived concentration of a substance that can be expected to cause death during a limited exposure interval (usually 4 hours) or within a fixed time after exposure in 50 percent of animals exposed when administered by inhalation. The LC50 value is expressed as weight or volume of test substance per standard volume of air (e.g. mg/l or ppm).

III. PRINCIPLE OF THE TEST METHOD

Several groups of experimental animals are exposed for a defined period to the test substance in graduated concentrations, one concentration being used per group. Subsequently observations of effects and deaths are made. Animals which die during the test should be necropsied and at the conclusion of the test surviving animals should be sacrificed and necropsied as necessary.

IV. LIMIT TEST

If a test at an exposure of 5 mg/l (actual concentration of respirable substances) for 4 hours or, where this is not possible due to physical or chemical properties of the test substance, the maximum attainable concentration, using the procedures described for this study, produces no compound-related mortality, then a full study using three dose levels might not be necessary.

V. TEST PROCEDURES

A. Animal Selection

1. Species and strain

Although several mammalian test species may be used the rat is the preferred species. Commonly used laboratory strains should be used. If another mammalian species is employed, the tester should provide justification/reasoning for its selection.

2. Age

Young adult animals should be used. The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight of each sex.

3. Sex

   a. Equal numbers of animals of each sex should be used for each dose level.

   b. The females should be nulliparous and non-pregnant.

4. Numbers

At least 10 animals (5 females and 5 males) at each dose level should be used.

B. Control groups

Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere a vehicle control group should be used.

C. Dose levels and dose selection

1. At least three exposure concentrations should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose-mortality curve and, where possible, permit an acceptable determination of an LC50.

2. Where necessary, a suitable vehicle may be added to the test substance to help generate an appropriate concentration of the test substance in the atmosphere. If a vehicle or diluent is needed, ideally it should not ellicit important toxic effects itself or substantially alter the chemical or toxicological properties of the test substance.

3. In the case of potentially explosive test substances, care should be taken to avoid generating explosive concentrations.

4. To establish suitable exposure concentrations, a trial test is recommended.

D. Exposure duration

The duration of exposure should be at least 4 hours after equilibration of the chamber concentrations.

E. Observation period

The observation period should be at least 14 days. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time of death are important, especially if there is a tendency for deaths to be delayed.

F. Inhalation exposure

1. The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 percent of the volume of the test chamber. Alternatively, oro-nasal, head-only, or whole body individual chamber exposure may be used.

2. A suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of a slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding area.

3. The temperature at which the test is performed should be maintained at 22°C (± 2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g. tests of aerosols, use of water vehicle) this may not be practicable.

G. Physical measurements

Measurements or monitoring should be made of the following:

1. The rate of air flow should be monitored continuously, but should be recorded at least every 30 minutes.

2. The actual concentrations of the test substance should be measured in the breathing zone. During the exposure period the actual concentration of the test substance should be held as constant as practicable. Continuous monitoring is desirable. Measurement of actual concentrations should be recorded near the beginning, middle, and end of the exposure period.

3. During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be made as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

4. Temperature and humidity should be monitored continuously but should be recorded at least every 30 minutes.

H. Food and water during exposure period

Food should be withheld during exposure. Water may also be withheld in certain cases.

I. Observation of animals

1. A careful clinical examination should be made at least once each day.

2. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g. necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals).

3. Cage-side observations should include, but not be limited to, changes in the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention should be directed to observation of tremors, convulsions, salivation,diarrhea, lethargy, sleep and coma.

4. Individual weights of animals should be determined shortly before the test substance is administered. Individual weights should be taken weekly thereafter and at death. Changes in weight should be calculated and recorded when survival exceeds one day.

5. The time of death should be recorded as precisely as possible.

6. At the end of the test, the surviving animals should be weighed and sacrificed.

J. Gross pathology

Consideration should be given to performing a gross necropsy of all animals where indicated by the nature of the toxic effects observed with particular reference to any changes in the respiratory tract. Where there are signficant signs of toxicity indicating the possible involvement of other organs, these should be examined and all gross pathological changes recorded.

K. Histopathology

Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 hours or more, should be considered since it may yield useful information.

VI. DATA AND REPORTING

A. Treatment of results

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, time of death of individual animals at different exposure levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings.

B. The LC50 value should always be considered in conjunction with the observed toxic effects and any necropsy findings. The LC50 value is a relatively coarse measurement, useful only as a reference value for classification and labelling purposes, and expressing possible lethal potential of the test substance following inhalation. Reference should always be made to the experimental animal species in which the LC50 value was obtained. An evaluation should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross lesions, body weight changes, effects on mortality and any other toxicological effects.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Test conditions

   a. Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

   b. The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described.

2. Exposure data

   These should be tabulated and presented with mean values and a measure of variablity (e.g. standard deviation) and should include:

   a. Airflow rates through th inhalation equipment;

   b. Temperature and humidity of air;

   c. Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air);

d. Actual concentration in test breathing zone; and

e. Particle size distribution (e.g. median aerodynamic diameter of particles with standard deviation from the mean)

3. Animal data

a. Tabulation of response data by sex and exposure level (i.e. number of animals dying, number of animals showing signs of toxicity, number of animals exposed);

b. Description of toxic effects;

c. Time of death during or following exposure;

d. LC50 for each sex determined at 14 days (with method of calculations specified);

e. Ninety-five percent confidence interval for the LC50;

f. Dose-mortality curve and slope (where permitted by the method of determination);

g. Body weight data; and

h. Pathology findings, when performed.

16

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Bliss, C.I. 1938. The determination of the dosage mortality curve from small numbers. Quarterly Journal Pharm. Pharmacology. 11:192-216.

2. Finney, D.G. 1971. Probit Analysis. Chapter 3--Estimation of the median effective dose, Chapter 4--Maximum likelihood estimation. 3rd Edition. London: Cambridge University Press. 60 pp.

3. Litchfield, J.T., Jr., Wilcoxon, F. 1949. A simplified method of evaluating dose-effect experiments. Journal of Pharmacology and Experimental Therapeutics. 96:99-115.

4. Miller, L.C., Tainter, M.L. 1944. Estimation of the ED50 and its error by means of logarithmic graph paper. Proceedings of the Society for Experimental Biology and Medicine. 57:261-264.

5. NAS. 1977. National Academy of Sciences. Principles and Procedures for Evaluating the Toxicity of Household Substances. Washington, D.C.: A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Reserach Council, National Academy of Sciences. 130 pp.

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8. Weil, C.S. 1952. Tables for convenient calculation of median effective dose and instructions in their use. Biometrics. 8:249-263.

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HG-Acute-Oral
1983

ACUTE EXPOSURE
ORAL TOXICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
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I. PURPOSE

In the assessment and evaluation of the toxic characteristics of a substance, determination of acute oral toxicity is usually an initial step. It provides information on health hazards likely to arise from a short term exposure by the oral route. The purpose of an acute oral study is to determine the median lethal dose (LD50), its statistical limits and slope using a single exposure or multiple doses given within to a 24-hour period and a 14-day post-exposure observation period. This purpose can be accomplished by performing the provisions contained in this guideline. Data from an acute study serves as a basis for classification and labelling. It is also an initial step in establishing a dosage regimen in subchronic and other studies. With the addition of certain other test elements, this guideline may provide information on the mode of toxic action of a substance.

II. DEFINITIONS

A. Acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours.

B. Dosage is a general term comprising the dose, its frequency and the duration of dosage.

C. Dose is the amount of test substance administered. Dose is expressed as weight of test substance (g, mg) per unit weight of test animal (e.g. mg/kg).

D. Dose-effect is the relationship between the dose and the magnitude of a defined biological effect either in an individual or in a population sample.

E. Dose-response is the relationship between the dose and the proportion of a population sample showing a defined effect.

F. LD50 (median lethal dose), oral, is a statistically derived single dose of a substance that can be expected to cause death in 50 percent of animals when administered by the oral route. The LD50 value is expressed in terms of weight of test substance (g, mg) per unit weight of test animal (e.g. mg/kg).

III. PRINCIPLE OF THE TEST METHOD

The test substance is administered orally by gavage in graduated doses to several groups of experimental animals, one dose being used per group. Subsequently observations of effects and deaths are made. Animals which die during the test should be necropsied, and at the conclusion of the test the surviving animals should be sacrificed and necropsied. This guideline is directed primarily to studies in rodent species but may be adapted for studies in non-rodents.

IV. LIMIT TEST

If a test at a dose level of at least 5000 mg/kg body weight, using the procedures described for the study, produces no compound-related mortality, then a full study using three dose levels might not be necessary.

V. TEST PROCEDURES

A. Animal selection

1. Species and strain

Although several mammalian test species may be used, the rat is the preferred species. Commonly used laboratory strains should be employed. If another species is used, the tester should provide justification and reasoning for its selection.

2. Age

Young adult animals should be used. The weight variation of animals used in a test should not exceed ± 20 percent of the mean weight for each sex.

3. Sex

a. Equal numbers of animals of each sex should be used for each dose level.

b. The females should be nulliparous and non-pregnant.

4. Numbers

At least 10 animals (5 females and 5 males) at each dose level should be used.

B. Control groups

Neither a concurrent untreated nor vehicle control group is required except when the toxicity of the vehicle is unknown.

C. Dose levels and dose selection

1. At least three dose levels should be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose response curve and, where possible, permit an acceptable determination of the LD50.

2. Vehicle

Where necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a solution in oil (e.g. corn oil) and then by possible solution in other vehicles. For non-aqueous vehicles the toxic characteristics of the vehicle should be known, and if not known should be determined before the test.

3. Volume

The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not exceed 1 ml/100 g body weight, except when an aqueous solution is used where 2 ml/100 g may be administered. Variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

D. Exposure duration

The test substance should be administered over a period not exceeding 24 hours.

E. Observation period

The observation period should be at least 14 days. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time to death are important, especially if there is a tendency for deaths to be delayed.

F. Exposure

1. The test substance should be administered in a single dose by gavage, using a stomach tube or suitable intubation cannula.

2. Animals should be fasted prior to test substance administration. For the rat, food should be withheld overnight; for other rodents with higher metabolic rates a shorter period of fasting is appropriate.

3. After the substance has been administered, food may be withheld for an additional 3-4 hours.

4. If a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. Where a dose is administered in fractions, it may be necessary to provide the animals with food and water depending on the length of the dosing period.

G. Observation of animals

1. A careful clinical examination should be made at least once each day.

2. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g. necropsy or refrigeration of those animals found dead and isolation of weak or moribund animals).

3. Cage-side observations should include, but not be limited to, changes in the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern. Particular attention should be directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

4. Individual weights of animals should be determined shortly before the test substance is administered, weekly thereafter and at death. Changes in weights should be calculated and recorded when survival exceeds one day.

5. The time of death should be recorded as precisely as possible.

6. At the end of the test, surviving animals should be weighed and sacrificed.

H. Gross pathology

Consideration should be given to performing a gross necropsy of all animals where indicated by the nature of the toxic effects observed. All gross pathology changes should be recorded.

I. Histopathology

Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 hours or more should also be considered because it may yield useful information.

VI. DATA AND REPORTING

A. Treatment of results

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, time of death of individual animals at different dose levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings.

B. Evaluation of results

The LD50 value should always be considered in conjunction with the observed toxic effects and any necropsy findings. The LD50 value is a relatively coarse measurement useful only as a reference value for classification and labelling purposes, and for expressing the possible lethal potential of the test substance by the injestion route. Reference should always be made to the experimental animal species in which the LD50 value was obtained. An evaluation should include the relationship, if any, between the animal's exposure to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxicological effects.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Tabulation of response data by sex and dose level (i.e. number of animals dying; number of animals showing signs of toxicity; number of animals exposed);
2. Description of toxic effects;
3. Time of death after dosing;
4. LD50 value for each sex determined at 14 days (with the method of determination specified);
5. Ninety-five percent confidence interval for the LD50;
6. Dose-mortality curve and slope (where permitted, by the method of determination);
7. Body weight data; and
8. Pathology findings, when performed.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Balazs, T. 1970. "Measurement of acute toxicity," in "Methods in Toxicology." Edited by G.E. Paget. Philadelphia: F.A. Davis Co. PP. 49-82.

2. Bliss, C.I. 1938. The determination of the dosage mortality curve from small numbers. Quarterly Journal Pharm. Pharmacology. 11:192-216.

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5. Litchfield, J.T., Jr., Wilcoxon, F. 1949. A simplified method of evaluating dose-effect experiments, Journal of Pharmacology and Experimental Therapeutics. 96:99-115.

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HG-Subchronic-Dermal
1983

SUBCHRONIC EXPOSURE
DERMAL TOXICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic dermal toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic dermal study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposure by the dermal route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

II. DEFINITIONS

A. Subchronic dermal toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by dermal application for part (approximately 10 percent) of a life span.

B. Dose in a dermal test is the amount of test substance applied to the skin (applied daily in subchronic tests). Dose is expressed as weight of the substance (g, mg) per unit weight of test animal (e.g. mg/kg).

C. No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed-effect level is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of the weight of a test substance given daily per unit weight of test animal (mg/kg).

D. Cumulative toxicity is the adverse effects of repeated doses occuring as a result of prolonged action on, or increased concentration of the administered test substance or its metabolites in susceptible tissues.

III. PRINCIPLE OF THE TEST METHOD

The test substance is applied daily to the skin in graduated doses to several groups of experimental animals, one dose level per unit group, for a period of 90 days. During the period of application the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied and appropriate histopathological examinations carried out.

IV. LIMIT TEST

If a test at one dose level of at least 1000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary.

V. TEST PROCEDURES

A. Animal selection

1. Species and strain

The rat, rabbit or guinea pig may be used although the albino rabbit is preferred. The albino rabbit is preferred because of its size, skin permeability and extensive data base. Commonly used laboratory strains should be employed. If another mammalian species is used, the tester should provide justification/reasoning for its selection.

2. Age

Young adult animals should be used. The following weight ranges at the start of the test are suggested in order to provide animals of a size which facilitates the conduct of the test: rats, 200 to 300 g; rabbits, 2.0 to 3.0 kg; guinea pigs, 350 to 450 g.

3. Sex

a. Equal numbers of animals of each sex with healthy skin should be used at each dose level.

b. The females should be nulliparous and non-pregnant.

4. Numbers

   a. At least 20 animals (10 females and 10 males) should be used at each dose level.

   b. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before completion of the study.

B. Control groups

A concurrent control group is recommended. This group should be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended.

C. Satellite group

A satellite group of 20 animals (10 animals per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurence, of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

D. Dose level and dose selection

1. In subchronic toxicity tests, it is desirable to have a dose-response relationship as well as a no-observed-toxic-effect level. Therefore, at least three dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) should be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a dose- response curve.

2. The highest dose level should result in toxic effects but not produce severe skin irritation or an incidence of fatalities which would prevent a meaningful evaluation.

3. The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure, the lowest dose level should exceed this.

4. Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

5. In the low and intermediate groups and in the controls the incidence of fatalities should be low, to permit a meaningful evaluation of the results.

E. Exposure conditions

The animals are treated with test substance, ideally for at least 6 hours per day on a 7-day per week basis, for a period of 90 days. However, based primarily on practical considerations, application on a 5-day per week basis is considered to be acceptable.

F. Observation period

1. Duration of observation should be for at least 90 days.

2. Animals in the satellite group scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects.

G. Preparation of animal skin

1. Shortly before testing, fur should be clipped from the dorsal area of the trunk of the test animals. Shaving may be employed, but it should be carried out approximately 24 hours before the test. Repeat clipping or shaving is usually needed at approximately weekly intervals. When clipping or shaving the fur, care should be taken to avoid abrading the skin, which could alter its permeability.

2. Not less than 10 percent of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of any covering used.

3. When testing solids, which may be pulverized if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence of the vehicle on toxicity of and penetration of the skin by the test substance should be taken into account.

H. Application of the test substance

1. The test substance should be applied uniformly over an area which is approximately 10 percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

2. During the exposure period, the test substance should be held in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilization is not a recommended method.

I. Observation of animals

1. Each animal should be handled and its physical condition appraised at least once each day.

2. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

3. Signs of toxicity should be recorded as they are observed, including the time of onset, the degree and duration.

4. Cage-side observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

5. Animals should be weighed weekly. Food consumption should also be determined weekly if abnormal body weight changes are observed.

6. At the end of the study period, all survivors in the non-satellite treatment groups are sacrificed. Moribund animals should be removed and sacrificed when noticed.

J. Clinical examinations

1. The following examinations should be made on at least 5 animals of each sex in each group:

   a. Certain hematology determinations should be carried out at least three times during the test period: just prior to initiation of dosing (baseline data), after approximately 30 days on test and just prior to terminal sacrifice at the end of the test period. Hematology determinations which should be appropriate to all studies: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

   b. Certain clinical biochemistry determinations on blood should be carried out at least three times: just prior to initiation of dosing (baseline data), after approximately 30 days on test and just prior to terminal sacrifice at the end of the test period. Test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with the period of fasting appropriate to the species), serum glutamic-pyruvic transaminase*, serum glutamic oxaloacetic transaminase**, ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include: analyses of lipids, hormones, acid/base balance, methemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

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* Now known as serum alanine aminotransferase.

** Now known as serum aspartate aminotransferase.

2. The following examinations should be made on at least 5 animals of each sex in each group:

   a. Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to exposure to the test substance and at the termination of the study. If changes in the eyes are detected all animals should be examined.

   b. Urinalysis is not suggested on a routine basis, but only when there is an indication based on expected or observed toxicity.

K. Gross necropsy

1. All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents.

2. The liver, kidneys, adrenals, brain and gonads should be weighed wet, as soon as possible after dissection, to avoid drying.

3. The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: normal and treated skin; all gross lesions; brain - including sections of medulla/pons, cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; lungs; heart; (sternum with bone marrow); salivary glands; liver; spleen; kidneys;

adrenals; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); aorta; gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve; (eye); (femur - including articular surface); (spinal cord at three levels - cervical; midthoracic and lumbar); and (exorbital lachrymal glands).

L. Histopathology

The following histopathology should be performed:

1. Full histopathology on normal and treated skin and on organs and tissues, listed above, of all animals in the control and high dose groups.

2. All gross lesions in all animals.

3. Target organs in all animals.

4. The tissues mentioned in brackets (listed above) - if indicated by signs of toxicity or expected target organ involvement.

5. Lungs of animals (rodents) in the low and intermediate dose groups should be subjected to histopathological examination for evidence of infection, since this provides a convenient assessment of the state of health of the animals.

6. When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in other treated groups.

VI. DATA AND REPORTING

A. Treatment of results

1. Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

2. All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods should be selected during the design of the study.

B. Evaluation of results

The findings of a subchronic dermal toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings. The evaluation should include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effect on mortality and any other general or specific toxic effects. A properly conducted subchronic test should provide a statisfactory estimation of a no-effect level.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported.

1. Group animal data

   Tabulation of toxic response data by species, strain, sex and exposure level for:

   a. Number of animals dying;

   b. Number of animals showing signs of toxicity; and

   c. Number of animals exposed.

2. Individual animal data

   a. Time of death during the study or whether animals survived to termination;

   b. Time of observation of each abnormal sign and its subsequent course;

   c. Body weight data;

   d. Food consumption data when collected;

   e. Hematological tests employed and all results;

f. Clinical biochemistry tests employed and all results;

g. Necropsy findings;

h. Detailed description of all histopathological findings; and

i. Statistical treatment of results where appropriate.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Draize, J.H. 1959. Third Printing: 1975. "Dermal toxicity," in "Appraisal of Chemicals in Food, Drugs and Cosmetics." The Association of Food and Drug Officials of the United States. PP. 46-59.

2. Fitzhugh, O.G. 1959. Third Printing: 1975. "Subacute toxicity," in "Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics." The Association of Food and Drug Officials of the United States. PP. 26-35.

3. NAS. 1977. National Academy of Sciences. Prinicples and Procedures for Evaluating the Toxicity of Household Substances. Washington, D.C.: A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences. 130 pp.

4. WHO. 1978. World Health Organization. Principles and Methods for Evaluating the Toxicity of Chemicals. Part I. Environmental Health Criteria 6. Geneva: World Health Organization. 272 pp.

HG-Subchronic-Inhal
1983

SUBCHRONIC EXPOSURE
INHALATION TOXICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

In the assessment and evaluation of the toxic characteristics of a gas, volatile substance, or aerosol/particulate, determination of subchronic inhalation toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic inhalation study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposures by the inhalation route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure. Hazards of inhaled substances are influenced by the inherent toxicity and by physical factors such as volatility and particle size.

II. DEFINITIONS

A. Subchronic inhalation toxicity is the adverse effects occuring as a result of the repeated daily exposure of experimental animals to a chemical by inhalation for part (approximately 10 percent) of a life span.

B. Aerodynamic diameter applies to the size of particles of aerosols. It is the diameter of a sphere of unit density which behaves aerodynamically as the particle of the test substance. It is used to compare particles of different size and densities and to predict where in the respiratory tract such particles may be deposited. This term is used in contrast to measured or geometric diameter which is representative of actual diameters which in themselves cannot be related to deposition within the respiratory tract.

C. The geometric mean diameter or the median diameter is the calculated aerodynamic diameter which divides the particles of an aerosol in half based on the weight of the particles. Fifty percent of the particles by weight will be larger than the median diameter and 50 percent of the particles will be smaller than the median diameter. The median diameter describes the particle size distribution of any aerosol based on the weight and size of the particles.

D. Inhalable diameter refers to that aerodynamic diameter of a particle which is considered to be inhalable for the organism. It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract from the trachea to the alveoli. For man, inhalable diameter is considered as 15 micrometers or less.

E. Dose refers to an exposure level. Exposure is expressed as weight or volume of test substance per volume of air (mg/l), or as parts per million (ppm).

F. No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed-effect level is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of weight or volume of test substance given daily per unit volume of air (mg/l or ppm).

G. Cumulative toxicity is the adverse effects of repeated doses occuring as a result of prolonged action on, or increased concentration of the administered substance or its metabolites in susceptible tissues.

III. PRINCIPLE OF THE TEST METHOD

Several groups of experimental animals are exposed daily for a defined period to the test substance in graduated concentrations, one concentration being used per group, for a period of 90 days. During the period of administration, the animals are observed daily to detect signs of toxicity. Animals which die during the test are necropsied and at the conclusion of the test, surviving animals are sacrificed and necropsied and appropriate histopathological examinations carried out.

IV. TEST PROCEDURES

A. Animal selection

1. Species and strain

A variety of rodent species may be used although the rat is the preferred species. Commonly used laboratory strains should be employed. If another mammalian species is used, the tester should provide justification/reasoning for its selection.

2. Age

Young adult animals should be used. At the commencement of the study the weight variation of animals should not exceed ± 20 percent of the mean weight for each sex.

3. Sex

a. Equal numbers of animals of each sex should be used at each dose level.

b. Females should be nulliparous and non-pregnant.

4. Numbers

a. At least 20 animals (10 females and 10 males) should be used for each test group.

b. If interim sacrifices are planned, the number of animals should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

B. Control groups

A concurrent control group is recommended. This group should be an untreated or sham treated control group. Except for treatment with the test substance, animals in the control group should be handled in a manner identical to the test group animals. Where a vehicle is used to help generate an appropriate concentration of the substance in the atmosphere, a vehicle control group should be used. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended.

C. Satellite group

A satellite group of 20 animals (10 animals per sex) may be treated with the high concentration level for 90 days and observed for reversibility, persistence, or delayed occurence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

D. Dose levels and dose selection

1. In subchronic toxicity tests, it is desirable to have a dose-response relationship as well as a no-observed-toxic-effect level. Therefore, at least three dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) should be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a dose-response curve.

2. The highest concentration should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation.

3. The lowest concentration should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest concentration should exceed this.

4. Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose level is used, the concentrations should be spaced to produce a gradation of toxic effects.

5. In the low and intermediate groups and in the controls the incidence of fatalities should be low, to permit a meaningful evaluation of the results.

6. In the case of potentially explosive test substances, care should be taken to avoid generating explosive concentrations.

E. Exposure conditions

The animals are exposed to the test substance, ideally for 6 hours per day on a 7-day per week basis, for a period of 90 days. However, based primarily on practical considerations, exposure on a 5-day per week basis is considered to be acceptable.

F. Observation period

1. Duration of observation should be for at least 90 days.

2. Animals in a satellite group scheduled for follow-up observations should be kept for an additional 28 days without treatment to detect recovery from, or persistence of, toxic effects.

G. Inhalation exposure

1. The animals should be tested in inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour and ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule, to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 percent of the volume of the test chamber. Oro-nasal or head-only exposure may be used if it is desirable to avoid concurrent exposure by the dermal or oral routes.

2. A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment exposure chamber are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

3. The temperature at which the test is performed should be maintained at 22° C (± 2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g. tests of aerosols, use of water vehicle) this may not be practicable.

H. Physical measurements

Measurements or monitoring should be made of the following:

1. The rate of air flow should be monitored continuously but should be recorded at least every 30 minutes.

2. The actual concentrations of the test substance should be measured in the breathing zone. During the exposure period the actual concentrations of the test substance should be held as constant as practicable, monitored continuously and measured at least at the beginning, at an intermediate time and at the end of the exposure period.

3. During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle size distribution.

4. Temperature and humidity should be monitored continuously but should be recorded at least every 30 minutes.

I. Food and water during exposure period

Food should be withheld during exposure. Water may also be withheld in certain cases.

J. Observation of animals

1. Each animal should be handled and its physical condition appraised at least once each day.

2. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

3. Signs of toxicity should be recorded as they are observed including the time of onset, the degree and duration.

4. Cage-side observations should include, but not be limited to, changes in the skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

5. Animals should be weighed weekly. Food consumption should also be determined weekly if abnormal body weight changes are observed.

6. At the end of the study period all survivors in the non-satellite treatment groups should be sacrificed. Moribund animals should be removed and sacrificed when noticed.

K. Clinical examinations

1. The following examinations should be made on at least 5 animals of each sex in each group:

   a. Certain hematology determinations should be carried out at least three times during the test period: just prior to initiation of dosing (base line data), after approximately 30 days on test and just prior to terminal sacrifice at the end of the test period. Hematology determinations which should be appropriate to all studies: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

   b. Certain clinical biochemistry determinations on blood should be carried out at least three times: just prior to initiation of dosing (base line data), after approximately 30 days on test and just prior to terminal sacrifice at the end of the test period. Clinical biochemical test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase*, serum glutamic-oxaloacetic transaminase**, ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include: analyses of lipids, hormones, acid/base balance, methemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed, where necessary, to extend the investigation of observed effects.

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* Now known as serum alanine aminotransferase.

** Now known as serum aspartate aminotransferase.

2. The following examinations should be made on at least 5 animals of each sex in each group:

   a. Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to exposure to the test substance and at the termination of the study. If changes in the eyes are detected, all animals should be examined.

   b. Urinalysis is not recommended on a routine basis, but only when there is an indication based on expected or observed toxicity.

L. Gross pathology

1. All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices and the cranial, thoracic and abdominal cavities and their contents.

2. At least the liver, kidneys, adrenals, brain, and gonads should be weighed wet, as soon as possible after dissection to avoid drying.

3. The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: all gross lesions; lungs - which should be removed intact, weighed and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain - including sections of medulla/pons cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); aorta; (skin); gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve; (eyes); (femur - cervical, midthoracic and lumbar); and (exorbital lachrymal glands).

M. Histopathology

The following histopathology should be performed:

1. Full histopathology on the respiratory tract and other organs and tissues, listed above, of all animals in the control and high dose groups.
2. All gross lesions in all animals.
3. Target organs in all animals.
4. The tissues mentioned in brackets (listed above) if indicated by signs of toxicity or target organ involvement.
5. Lungs of animals in the low and intermediate dose groups should also be subjected to histopathological examination, primarily for evidence of infection since this provides a covenient assessment of the state of health of the animals.
6. When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in other treated groups.

V. DATA AND REPORTING

A. Treatment of results

1. Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions, the percentage of animals displaying each type of lesion.
2. All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods should be selected during the design of the study.

B. Evaluation of results

The findings of a subchronic inhalation toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the concentration of the test substance and duration of exposure, and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Test conditions

a. Description of exposure apparatus, including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing animals in a test chamber.

b. The equipment for measuring temperature, humidity and particulate aerosol concentrations and size should be described.

2. Exposure data

These should be tabulated and presented with mean values and measure of variability (e.g. standard deviation) and should include:

a. Airflow rates through the inhalation equipment;

b. Temperature and humidity of air;

c. Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air);

d. Actual concentration in test breathing zone; and

e. Particle size distribution (e.g. median aerodynamic diameter of particles with standard deviation from the mean).

3. Group animal data

Tabulation of toxic response data by species, strain, sex, and exposure level for:

a. Number of animals dying;

b. Number of animals showing signs of toxicity; and

c. Number of animals exposed.

4. Individual animal data

a. Time of death during the study or whether animals survived to termination;

b. Time of observation of each abnormal sign and its subsequent course;

c. Body weight data;

d. Food consumption data when collected;

e. Hematological tests employed and all results;

f. Clinical biochemistry tests employed and all results;

g. Necropsy findings;

h. Detailed description of all histopathological findings; and

i. Statistical treatment of results where appropriate.

VI. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Cage, J.C. 1970. "Experimental Inhalation Toxicology," in "Methods in Toxicology." Edited by G.E. Paget. Philadelphia: F.A. Davis Company. PP. 258-277.

2. Casarett, L.J., Doull, J. 1975. Toxicology: The Basic Science of Poisons. New York: Macmillan Publishing Co. Inc. Chapter 9.

3. MacFarland, H.N. 1976. "Respiratory Toxicology," in "Essays in Toxicology." Edited by W.J. Hayes. New York: Academic Press. Vol. 7. PP. 121-154.

4. NAS. 1977. National Academy of Sciences. Principles and Procedures for Evaluating the Toxicity of Household Substances. Washington, D.C.: A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences. 130 pp.

5. WHO. 1978. World Health Organization. Principles and Methods for Evaluating the Toxicity of Chemicals. Part I. Environmental Health Criteria 6. Geneva: World Health Organization. 272 pp.

HG-Subchronic-Oral
1983

SUBCHRONIC EXPOSURE
ORAL TOXICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

In the assessment and evaluation of the toxic characteristics of a chemical, the determination of subchronic oral toxicity may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic oral study has been designed to permit the determination of the no-observed-effect level and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g., carcinogenicity and life shortening). It provides information on health hazards likely to arise from repeated exposure by the oral route over a limited period of time. It will provide information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

II. DEFINITIONS

A. Subchronic oral toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the oral route for a part (approximately ten percent for rats) of a life span.

B. Dose is the amount of test substance administered. Dose is expressed as weight of test substance (g, mg) per unit weight of test animal (e.g., mg/kg), or as weight of test substance per unit weight of food or drinking water.

C. No-effect level/No-toxic-effect level/No-adverse-effect level/No-observed-effect level is the maximum dose used in a test which produces no observed adverse effects. A no-observed-effect level is expressed in terms of the weight of a substance given daily per unit weight of test animal (mg/kg). When administered to animals in food or drinking water the no-observed-effect level is expressed as mg/kg of food or mg/ml of water.

D. Cumulative toxicity is the adverse effects of repeated doses occuring as a result of prolonged action on, or increased concentration of the administered substance or its metabolites in susceptible tissue.

III. PRINCIPLE OF THE TEST METHOD

The test substance is administered orally in graduated daily doses to several groups of experimental animals, one dose level per group, for a period of 90 days. During the period of administration the animals are observed daily to detect signs of toxicity. Animals which die during the period of administration are necropsied. At the conclusion of the test all animals are necropsied and histopathological examinations carried out.

IV. LIMIT TEST

If a test at one dose level of at least 1,000 mg/kg body weight (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary.

V. TEST PROCEDURES

A. Animal selection

1. Species and strain

A variety of rodent species may be used, although the rat is the preferred species. Commonly used laboratory strains should be employed. The commonly used non-rodent species is the dog, preferably of a defined breed; the beagle is frequently used. If other mammalian species are used, the tester should provide justification/reasoning for their selection.

2. Age

a. General

Young adult animals should be employed. At the commencement of the study the weight variation of animals used should not exceed ± 20 percent of the mean weight for each sex.

b. Rodents

Dosing should begin as soon as possible after weaning, ideally before the rats are 6, and in any case, not more than 8 weeks old.

c. Non-rodent

In the case of the dog, dosing should commence after acclimatization, preferably at 4-6 months and not later than 9 months of age.

3. Sex

a. Equal numbers of animals of each sex should be used at each dose level.

b. The females should be nulliparous and non-pregnant.

4. Numbers

a. Rodents

At least 20 animals (10 females and 10 males) should be used at each dose level.

b. Non-rodents

At least eight animals (4 females and 4 males) should be used at each dose level.

c. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

B. Control groups

A concurrent control group is recommended. This group should be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended.

C. Satellite group

(Rodent) A satellite group of 20 animals (10 animals per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

D. Dose levels and dose selection

1. In subchronic toxicity tests, it is desirable to have a dose response relationship as well as no-observed-toxic-effect level. Therefore, at least three dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) should be used. Doses should be spaced appropriately to produce test groups with a range ot toxic effects. The data should be sufficient to produce a dose response curve.

2. The highest dose level in rodents should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation; for non-rodents there should be no fatalities.

3. The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest dose level should exceed this.

4. Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

5. For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low, to permit a meaningful evaluation of the results; for non-rodents, there should be no fatalities.

E. Exposure conditions

The animals are dosed with the test substance ideally on a 7-day per week basis over a period of 90 days. However, based primarily on practical considerations, dosing in gavage or capsule studies on a 5-day per week basis is considered to be acceptable.

F. Observation period

1. Duration of observation should be for at least 90 days.

2. Animals in the satellite group scheduled for follow-up observations should be kept for a further 28 days without treatment to detect recovery from, or persistence of, toxic effects.

G. Administration of the test substance

1. The test substance may be administered in the diet or in capsules. In addition, for rodents it may also be administered by gavage or in the drinking water.

2. All animals should be dosed by the same method during the entire experimental period.

3. Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, ideally it should not elicit important toxic effects itself nor substantially alter the chemical or toxicological properties of the test substance. It is recommended that wherever possible the usage of an aqueous solution be considered first, followed by consideration of a solution of oil and then by possible solution in other vehicles.

4. For substances of low toxicity, it is important to ensure that when administered in the diet the quantities of the test substance involved do not interfere with normal nutrition. When the test substance is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used should be specified.

5. For a substance administered by gavage or capsule, the dose should be given at similar times each day, and adjusted at intervals (weekly or bi-weekly) to maintain a constant dose level in terms of animal body weight.

H. Observation of animals

1. Each animal should be handled and its physical condition appraised at least once each day.

2. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

3. Signs of toxicity should be recorded as they are observed including the time of onset, degree and duration.

4. Cage-side observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

5. Measurements should be made weekly of food consumption or water consumption when the test substance is administered in the food or drinking water, respectively.

6. Animals should be weighed weekly.

7. At the end of the 90-day period all survivors in the non-satellite treatment groups are sacrificed. Moribund animals should be removed and sacrificed when noticed.

I. Clinical examinations

1. The following examinations should be made on at least five animals of each sex in each group for rodents and all animals when non-rodents are used as test animals.

   a. Certain hematology determinations should be carried out at least three times during the test period: just prior to initiation of dosing (baseline data), after approximately 30 days on test and just prior to terminal sacrifice at the end of the test period. Hematology determinations which should be appropriate to all studies: hematocrit, hemoglobin concentration, erythrocyte count, total and differential leucocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count.

b. Certain clinical biochemistry determinations should be carried out at least three times during the test period: just prior to initiation of dosing (baseline data), after approximately 30 days on test and just prior to terminal sacrifice at the end of the test period. Clinical biochemical test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. Suggested determinations: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species/breed), serum glutamic-pyruvic transaminase*, serum glutamic oxaloacetic transaminase**, ornithine decarboxylase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects. Non-rodents should be fasted for a period (not more than 24 hours) before taking blood samples.

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* Now knowm as serum alanine aminotransferase.

** Now known as serum aspartate aminotransferase.

2. The following examinations should be made on at least five animals of each sex in each group for rodents and all animals on test for non-rodents.

a. Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study. If changes in the eyes are detected all animals should be examined.

b. Urinalysis is not recommmended on a routine basis, but only when there is an indication based on expected or observed toxicity.

J. Gross necropsy

1. All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents.

2. At least the liver, kidneys, adrenals, and gonads should be weighed wet, as soon as possible after dissection to avoid drying. In addition, for the rodent, the brain; for the non-rodent, the thyroid with parathyroids also should be weighed wet.

3. The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: all gross lesions; brain-including sections of medulla/pons, cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys/adrenals; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); aorta; (skin), (non-rat gall bladder); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland), (thigh musculature), peripheral nerve; (eyes); (femur-including articular surface);(spinal cord at three levels - cervical, midthoracic and lumbar); and, (rodent - exorbital lachrymal glands).

K. Histopathology

The following histopathology should be performed:

1. Full histopathology on the organs and tissues, listed above, of all rodents in the control and high dose groups, all non-rodents, and all rodents that died or were killed during the study.

2. All gross lesions in all animals.

3. Target organs in all animals.

4. The tissues mentioned in brackets (listed above) if indicated by signs of toxicity or target organ involvement.

5. Lungs, liver and kidneys of all animals. Special attention to examination of the lungs of rodents should be made for evidence of infection since this provides a convenient assessment of the state of health of the animals.

6. When a satellite group is used (rodents), histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

VI. DATA AND REPORTING

A. Treatment of results

1. Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

2. All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

B. Evaluation of the study results

1. The findings of a subchronic oral toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the dose of the test substance and the presence or absence, the incidence and severity, of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level.

2. In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Group animal data

Tabulation of toxic response data by species, strain, sex and exposure level for:

a. Number of animals dying;

b. Number of animals showing signs of toxicity; and

c. Number of animals exposed.

2. Individual animal data

a. Time of death during the study or whether animals survived to termination;

b. Time of observation of each abnormal sign and its subsequent course;

c. Body weight data;

d. Food consumption data when collected;

e. Hematological tests employed and all results;

f. Clinical biochemistry tests employed and all results;

g. Necropsy findings;

h. Detailed description of all histopathological findings; and

i. Statistical treatment of results where appropriate.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Boyd, E.M. 1972. Predictive Toxicometrics. Chapter 14--Pilot Studies, 15--Uniposal Clinical Parameters, 16--Uniposal Autopsy Parameters. Baltimore: Williams and Wilkins. 48 pp.

2. Fitzhugh, O.G. 1959. Third Printing: 1975. "Subacute Toxicity" in "Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics." The Association of Food and Drug Officials of the United States. PP. 26-35.

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CHRONIC EXPOSURE
CHRONIC TOXICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
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I. PURPOSE

The objective of a chronic toxicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. Under the conditions of the chronic toxicity test, effects which require a long latency period or which are cumulative should become manifest. The application of this guideline should generate data on which to identify the majority of chronic effects and shall serve to define long term dose-response relationships. The design and conduct of chronic toxicity tests should allow for the detection of general toxic effects, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects.

II. TEST PROCEDURES

A. Animal selection

1. Species and strain

Testing should be performed with two mammalian species, one a rodent and another a non-rodent. The rat is the preferred rodent species and the dog is the preferred non-rodent species. Commonly used laboratory strains should be employed. If other mammalian species are used, the tester should provide justification/reasoning for their selection.

2. Age

a. Dosing of rats should begin as soon as possible after weaning, ideally before the rats are six, but in no case more than eight weeks old.

b. Dosing of dogs should begin between four and six months of age and in no case later than nine months of age.

c. At commencement of the study the weight variation of animals used should not exceed ± 20 percent of the mean weight for each sex.

3. Sex

   a. Equal numbers of animals of each sex should be used at each dose level.

   b. The females should be nulliparous and non-pregnant.

4. Numbers

   a. For rodents, at least 40 animals (20 females and 20 males) and for non-rodents (dogs) at least eight animals (four females and four males) should be used at each dose level.

   b. If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed during the course of the study.

   c. The number of animals at the termination of the study must be adequate for a meaningful and valid statistical evaluation of chronic effects.

B. Control groups

1. A concurrent control group is suggested. This group should be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are strongly suggested.

2. In special circumstances such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should be utilized. The negative control group should be treated in the same manner as all other test animals except that this control group should not be exposed to either the test substance or any vehicle.

C. Dose levels and dose selection

I. In chronic toxicity tests, it is necessary to have a dose-response relationship as well as a no-observed-toxic-effect level. Therefore, at least three dose levels should be used in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of effects.

2. The high dose level in rodents should elicit some signs of toxicity without causing excessive lethality; for non-rodents, there should be signs of toxicity but there should be no fatalities.

3. The lowest dose level should not produce any evidence of toxicity. Where there is a usable estimation of human exposure the lowest dose level should exceed this even though this dose level may result in some signs of toxicity.

4. Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

5. For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low to permit a meaningful evaluation of the results. For non-rodents, there should be no fatalities.

D. Exposure conditions

The animals are dosed with the test substance ideally on a 7-day per week basis over a period of at least 12 months. However, based primarily on practical considerations, dosing on a 5-day per week basis is considered to be acceptable.

E. Observation period

Duration of observation should be for at least 12 months, and may be concurrent with or subsequent to dosing. If there is an post-exposure observation period, an interim sacrifice should be performed on no fewer than half of the animals of each sex at each dose level immediately upon termination of exposure.

F. Administration of the test substance

The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

1. Oral studies

a. The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 12 months.

b. If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

c. For a diet mixture, the highest concentration should not exceed 5 percent.

2. Dermal studies

a. The animals are treated by topical application with the test substance, ideally for at least 6 hours per day.

b. Fur should be clipped from the dorsal area of the trunk of the test animals. Care must be taken to avoid abrading the skin which could alter its permeability.

c. The test substance should be applied uniformly over a shaved area which is approximately ten percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

d. During the exposure period, the test substance may be held if necessary, in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance.

3. Inhalation studies

a. The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed 5 percent of the volume of the test chamber. Alternatively, oro-nasal, head-only or whole body individual chamber exposure may be used.

b. The temperature at which the test is performed should be maintained at 22°C (± 2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g. tests of aerosols, use of water vehicle) this may not be practicable.

c. Food and water should be withheld during each daily six-hour exposure period.

d. A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

G. Observation of animals

1. Each animal should be handled and its physical condition appraised at least once each day.

2. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

3. Clinical signs of toxicity including suspected tumors and mortality should be recorded as they are observed, including the time of onset, the degree and duration.

4. Cage-side observations should include, but not be limited to, changes in skin and fur, eyes and mucous membranes, respiratory, ciculatory, autonomic and central nervous systems, somatomotor activity and behavior pattern.

5. Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter unless signs of clinical toxicity suggest more frequent weighings to facilitate monitoring of health status.

6. When the test substance is administered in the food or drinking water, measurements of food or water consumption, respectively, should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

7. At the end of the study period all survivors should be sacrificed. Moribund animals should be removed and sacrificed when noticed.

H. Physical measurements

For inhalation studies, measurements or monitoring should be made of the following:

1. The rate of air flow should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

2. During each exposure period the actual concentrations of the test substance should be held as constant as practicable, monitored continuously and measured at least three times during the test period: at the beginning, at an intermediate time and at the end of the period.

3. During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analysis should be conducted as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

4. Temperature and humidity should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

I. Clinical examinations

The following examinations should be made on at least ten rats of each sex per dose and on all non-rodents.

1. Certain hematology determinations (e.g., hemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at termination and should be performed at three months, six months and at approximately six-month intervals thereafter (for studies extending beyond 12 months) on blood samples collected from all non-rodents and from ten rats per sex of all groups. These collections should be from the same animals at each interval. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed. A differential blood count should be performed on samples from those animals in the highest dosage group and the controls. Differential blood counts should be performed for the next lower group(s) if there is a major discrepancy between the highest group and the controls. If hematological effects were noted in the subchronic test, hematological testing should be performed at 3, 6, 12, 18 and 24 months for a two year study and at 3, 6 and 12 months for a one year study.

2. Certain clinical biochemistry determinations on blood should be carried out at least three times during the test period: just prior to initiation of dosing (base line data), near the middle and at the end of the test period. Blood samples should be drawn for clinical chemistry measurements from all non-rodents and at least ten rodents per sex of all groups; if possible, from the same rodents at each time interval. Test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity. Suggested chemical determinations: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase*, serum glutamic oxaloacetic transaminase**, ornithine decarboxylase, gamma glutamyl transpeptidase, blood urea nitrogen, albumen, blood creatinine, creatinine phosphokinase, total cholesterol, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.

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* Now known as serum alanine aminotransferase.
** Now known as serum aspartate aminotransferase.

3. Urine samples from rodents at the same intervals as the hematological examinations (above) should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group for rodents: appearance (volume and specific gravity), protein, glucose, ketones, bilirubin occult blood (semi-quantitatively); and microscopy of sediment (semi-quantitatively).

4. Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study. If changes in eyes are detected all animals should be examined.

J. Gross necropsy

1. A complete gross examination should be performed on all animals, including those which died during the experiment or were killed in moribund conditions.

2. The liver, kidneys, adrenals, brain and gonads should be weighed wet, as soon as possible after dissection to avoid drying. For these organs, at least ten rodents per sex per group and all non-rodents should be weighed.

3. The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: all gross lesions and tumors; brain - including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; sternum and/or femur with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles; female mammary gland; aorta; gall bladder (if present); skin; musculature; peripheral nerve; spinal cord at three levels-cervical, midthoracic, and lumbar; and eyes. In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx, and paranasal sinuses should be examined and preserved. In dermal studies, skin from sites of skin painting should be examined and preserved.

4. Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues. The proper inflation and fixation of the lungs in inhalation studies is considered essential for appropriate and valid histopathological examination.

5. If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

K. Histopathology

1. The following histopathology should be performed:

   a. Full histopathology on the organs and tissues, listed above, of all non-rodents, of all rodents in the control and high dose groups and of all rodents that died or were killed during the study.

   b. All gross lesions in all animals.

   c. Target organs in all animals.

   d. Lungs, liver and kidneys of all animals. Special attention to examination of the lungs of rodents should be made for evidence of infection since this provides an assessment of the state of health of the animals.

2. If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

3. In case the results of an experiment give evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a toxic response, the next lower dose level should be examined fully, as described above.

4. An attempt should be made to correlate gross observations with microscopic findings.

III. DATA AND REPORTING

A. Treatment of results

1. Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

2. All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

B. Evaluation of study results

1. The findings of a chronic toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation will include the relationship between the dose of the test substance and the presence, incidence and severity of abnormalities (including behavioral and clinical abnormalities), gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects.

2. In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Group animal data

Tabulation of toxic response data by species, strain, sex and exposure level for:

a. Number of animals dying;

b. Number of animals showing signs of toxicity; and

c. Number of animals exposed.

2. Individual animal data

a. Time of death during the study or whether animals survived to termination;

b. Time of observation of each abnormal sign and its subsequent course;

c. Body weight data;

d. Food and water consumption data, when collected;

e. Results of ophthalmological examination, when performed;

f. Hematological tests employed and all results;

g. Clinical biochemistry tests employed and all results;

h. Necropsy findings;

i. Detailed description of all histopathological findings; and

j. Statistical treatment of results, where appropriate.

In addition, for inhalation studies the following should be reported:

3. Test conditions

a. Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

b. The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described.

4. Exposure data

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

a. Airflow rates through the inhalation equipment;

b. Temperature and humidity of air;

c. Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air);

d. Actual concentration in test breathing zone; and

e. Particle size distribution (e.g. median aerodynamic diameter of particles with standard deviation from the mean).

IV. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

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1983

ONCOGENICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

The objective of a long-term oncogenicity study is to observe test animals for a major portion of their life span for the development of neoplastic lesions during or after exposure to various doses of a test substance by an appropriate route of administration.

II. TEST PROCEDURES

A. Animal selection

1. Species and strain

It is recommended that a compound of unknown activity should be tested on two mammalian species. Rats and mice are the species of choice because of their relatively short life spans, the limited cost of their maintenance, their widespread use in pharmacological and toxicological studies, their susceptibility to tumor induction, and the availability of inbred or sufficiently characterized strains. Commonly used laboratory strains should be employed. If other species are used, the tester should provide justification/reasoning for their selection.

2. Age

a. Dosing of rodents should begin as soon as possible after weaning, ideally before the animals are six, but in no case more than eight weeks old.

b. At commencement of the study, the weight variation of animals used should not exceed ± 20 percent of the mean weight for each sex.

c. Studies using prenatal or neonatal animals may be recommended under special conditions.

3. Sex

a. Equal numbers of animals of each sex should be used at each dose level.

b. The females should be nulliparous and non-pregnant.

4. Numbers

a. For rodents, at least 100 animals (50 females and 50 males) should be used at each dose level and concurrent control.

b. If interim sacrifices are planned the number should be increased by the number of animals scheduled to be sacrificed during the course of the study.

c. The number of animals at the termination of the study should be adequate for a meaningful and valid statistical evaluation of long term exposure. For a valid interpretation of negative results, it is essential that survival in all groups does not fall below 50 percent at the time of termination.

B. Control groups

1. A concurrent control group is recommended. This group should be an untreated or sham treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended.

2. In special circumstances such as in inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should be utilized. The negative control group should be treated in the same manner as all other test animals except that this control group should not be exposed to either the test substance or any vehicle.

3. The use of historical control data (i.e., the incidence of tumors and other suspect lesions normally occuring under the same laboratory conditions and in the same strain of animals employed in the test) is desirable for assessing the significance of changes observed in exposed animals.

C. Dose levels and dose selection

1. For risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of chronic effects.
2. The high dose level should elicit signs of minimal toxicity without substantially altering the normal life span.
3. The lowest dose should not interfere with normal growth, development and longevity of the animal; and it should not otherwise cause any indication of toxicity. In general, this should not be lower than ten percent of the high dose.
4. The intermediate dose(s) should be established in a mid-range between the high and low doses, depending upon the toxicokinetic properties of the chemical, if known.
5. The selection of these dose levels should be based on existing data, preferably on the results of subchronic studies.

D. Exposure conditions

The animals are dosed with the test substance ideally on a seven-day per week basis over a period of at least 24 months for rats, and 18 months for mice. However, based primarily on practical considerations, dosing on a five-day per week basis is considered to be acceptable.

E. Observations period

It is necessary that the duration of an oncogenicity test comprise the majority of the normal life span of the strain of animals to be used. This time period should not be less than 24 months for rats and 18 months for mice, and ordinarily not longer than 30 months for rats and 24 months for mice. For longer time periods, and where any other species are used, consultation with the Agency in regard to the duration of the test is advised.

F. Administration of the test substance

The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

1. Oral studies

a. The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 24 months for rats and 18 months for mice.

b. If the test substance is administered in the drinking water, or mixed in the diet, exposure should be continuous.

c. For a diet mixture, the highest concentration should not exceed five percent.

2. Dermal studies

a. The animals are treated by topical application with the test substance, ideally for at least six hours per day.

b. Fur should be clipped from the dorsal area of the trunk of the test animals. Care should be taken to avoid abrading the skin which could alter its permeability.

c. The test substance should be applied uniformly over a shaved area which is approximately ten percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

d. During the exposure period, the test substance may be held, if necessary, in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance.

3. Inhalation studies

a. The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed five percent of the volume of the test chamber. Alternatively, oro-nasal, head-only, or whole body individual chamber exposure may be used.

b. The temperature at which the test is performed should be maintained at 22°C (± 2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g. tests of aerosols, use of water vehicle) this may not be practicable.

c. Food and water should be withheld during each daily six-hour exposure period.

d. A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

G. Observation of animals

1. Each animal should be handled and its physical condition appraised at least once each day.

2. Additional observations should be made daily with appropriate actions take to minimize loss of animals to the study (e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

3. Clinical signs and mortality should be recorded for all animals. Special attention should be paid to tumor development. The time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded.

4. Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter unless signs of clinical toxicity suggest more frequent weighings to facilitate monitoring of health status.

5. When the test substance is administered in the food or drinking water, measurements of food or water consumption, respectively, should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

6. At the end of the study period all survivors are sacrificed. Moribund animals should be removed and sacrificed when noticed.

H. Physical measurements

For inhalation studies, measurements or monitoring should be made of the following:

1. The rate of air flow should be monitored continuously, but should be recorded at intervals of at least once very 30 minutes.

2. During each exposure period the actual concentrations of the test substance should be held as constant as practicable, monitored continuously and measured at least three times during the test period: at the beginning, at an intermediate time and at the end of the period.

3. During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analyses should be conducted as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

4. Temperature and humidity should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

I. Clinical examinations

At 12 months, 18 months and at sacrifice, a blood smear should be obtained from all animals. A differential blood count should be performed on blood smears from those animals in the highest dosage group and the controls. If these data, or data from the pathological examiniation indicate a need, then the 12 and 18 month blood smears from other dose levels should also be examined. Differential blood counts should be performed for the next lower group(s) only if there is a major discrepancy between the highest group and the controls. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed.

J. Gross necropsy

1. A complete gross examination should be performed on all animals, including those which died during the experiment or were killed in moribund conditions.

2. The following organs and tissues or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: all gross lesions and tumors of all animals should be preserved; brain - including sections of medulla/pons, cerebellar cortex and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; spinal cord at three levels - cervical, midthoracic and lumbar; sternum and/or femur with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; pancreas; gonads;

uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); female mammary gland; skin; musculature; peripheral nerve; and eyes. In special studies such as inhalation studies, the entire respiratory tract should be preserved, including nasal cavity, pharynx, larynx and paranasal sinuses. In dermal studies, skin from sites of skin painting should be examined and preserved.

3. Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues. The proper inflation and fixation of the lungs in inhalation studies is a necessary requirement for appropriate and valid histopathological examination.

4. If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

K. Histopathology

1. The following histopathology should be performed:

   a. Full histopathology on organs and tissues listed above of all animals in the control and high dose groups and all animals that died or were killed during the study.

   b. All gross lesions in all animals.

   c. Target organs in all animals.

2. If a significant difference is observed in hyperplastic, pre-neoplastic or neoplastic lesions between the highest dose and control groups, microscopic examination should be made on that particular organ or tissue of all animals in the study;

3. If excessive early deaths or other problems occur in the high dose group, compromising the significance of the data, the next lower dose level should be examined for complete histopathology.

4. In case the results of an experiment give evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a neoplastic response, the next lower dose level should be examined fully as described above.

5. An attempt should be made to correlate gross observations with microscopic findings.

III. DATA AND REPORTING

A. Treatment of results

1. Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

2. All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

B. Evaluation of study results

1. The findings of an oncogenic toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation should include the relationship between the dose of the test substance and the presence, incidence and severity of abnormalities (including behavioral and clinical abnormalities), gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects.

2. In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

3. In order for a negative test to be acceptable, it should meet the following criteria: no more than ten percent of any group is lost due to autolysis, cannibalism, or management problems; and survival in each group is no less than 50 percent at 18 months for mice and hamsters and at 24 months for rats.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Group animal data

Tabulation of toxic response data by species, strain, sex and exposure level for:

a. Number of animals dying;

b. Number of animals showing signs of toxicity; and

c. Number of animals exposed.

2. Individual animal data

a. Time of death during the study or whether animals survived to termination;

b. Time of observation of each abnormal sign and its subsequent course;

c. Body weight data;

d. Food and water consumption data, when collected;

e. Results of ophthalmological examination, when performed;

f. Hematological tests employed and all results;

g. Clinical biochemistry tests employed and all results;

h. Necropsy findings;

i. Detailed description of all histopathological findings;

j. Statistical treatment of results, where appropriate; and

k. Historical control data, if taken into account.

In addition, for inhalation studies the following should be reported:

3. Test conditions

a. Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

b. The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described.

4. Exposure data

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

a. Airflow rates through the inhalation equipment;

b. Temperature and humidity of air;

c. Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air);

d. Actual concentration in test breathing zone; and

e. Particle size distribution (e.g. median aerodynamic diameter of particles with standard deviation from the mean).

IV. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. DHEW. 1975. Department of Health and Welfare. The Testing of Chemicals for Carcinogenicity, Mutagenicity, Teratogenicity. Canada: The Honorable marc Lalonde, Minister of Health and Welfare. Department of Health and Welfare. 183 pp.

2. Food and Drug Administration Advisory Committee on Protocols for Safety Evaluation: Panel on Carcinogenesis. 1971. Report on Cancer Testing in the Safety of Food Additives and Pesticides. Toxicology and Applied Pharmacology. 20:419-438.

3. IUCC. 1969. International Union Against Cancer. "Carcinogenicity Testing," in "IUCC Technical Report Series, Vol. 2," Edited by I. Berenblum. Geneva: International Union Against Cancer. 56 pp.

4. Leong, B.K.J., Laskin, S. 1975. Number and Species of Experimental Animals for Inhalation Carcinogenicity Studies. Cincinnati: Paper presented at Conference on Target Organ Toxicity. Sept., 1975.

5. NAS. 1977. National Academy of Sciences. Prinicples and Procedures for Evaluating the Toxicity of Household Substances. Washington, D.C.: A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences. 130 pp.

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8. Page, N.P. 1977. Chronic Toxicity and Carcinogenicity Guidelines. Journal Environmental Pathology and Toxicology. 1:161-182.

9. Page, N.P. 1977. "Concepts of a Bioassay Program in Environmental Carcinogenesis," in "Advances in Modern Toxicology." Edited by Kraybill and Mehlman. Washington, D.C.: Hemisphere Publishing Corporation. Volume 3. PP. 87-171.

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HG-Chronic-Combined
1983

COMBINED CHRONIC TOXICITY/ONCOGENICITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

The objective of a combined chronic toxicity/oncogenicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. The application of this guideline should generate data which identify the majority of chronic and oncogenic effects and determine dose-response relationships. The design and conduct should allow for the detection of neoplastic effects and a determination of oncogenic potential as well as general toxicity, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathology). effects.

II. TEST PROCEDURES

A. Animal selection

1. Species and strain

Preliminary studies providing data on acute, subchronic, and metabolic responses should have been carried out to permit an appropriate choice of animals (species and strain). As discussed in other guidelines, the mouse and rat have been most widely used for assessment of oncogenic potential, while the rat and dog have been most often studies for chronic toxicity. The rat is the species of choice for combined chronic toxicity and oncogenicity studies. The provisions of this guideline are designed primarily for use with the rat as the test species. If other species are used, the tester should provide justification/ reasoning for their selection. The strain selected should be susceptible to the oncogenic or toxic effect of the class of substances being tested, if known, and provided it does not have a spontaneous background too high for meaningful assessment. Commonly used laboratory strains should be employed.

2. Age

a. Dosing of rats should begin as soon as possible after weaning, ideally before the rats are six, but in no case more than eight weeks old.

b. At commencement of the study, the weight variation of animals used should exceed ± 20 percent of the mean weight for each sex.

c. Studies using prenatal or neonatal animals may be recommended under special conditions.

3. Sex

a. Equal numbers of animals of each sex should be used at each dose level.

b. The females should be nulliparous and non-pregnant.

4. Numbers

a. At least 100 rodents (50 females and 50 males) should be used at each dose level and concurrent control for those groups not intended for early sacrifice. At least 40 rodents (20 females and 20 males) should be used for satellite dose group(s) and the satellite control group. The purpose of the satellite group is to allow for the evaluation of pathology other than neoplasia.

b. If interim sacrifices are planned, the number of animals should be increased by the number of animals scheduled to be sacrificed during the course of the study.

c. The number of animals at the termination of each phase of the study should be adequate for a meaningful and valid statistical evaluation of long term exposure. For a valid interpretation of negative results, it is essential that survival in all groups does not fall below 50 percent at the time of termination.

B. Control groups

1. A concurrent control group (50 females and 50 males) and a satellite control group (20 females and 20 males) are recommended. These groups should be untreated or sham treated control groups or, if a vehicle is used in administering the test substance, vehicle control groups. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are recommended. Animals in the satellite control group should be sacrificed at the same time the satellite test group is terminated.

2. In special circumstances such as inhalation studies involving aerosols or the use of an emulsifier of uncharacterized biological activity in oral studies, a concurrent negative control group should be utilized. The negative control group should be treated in the same manner as all other test animals, except that this control group should not be exposed to the test substance or any vehicle.

3. The use of historical control data (i.e., the incidence of tumors and other suspect lesions normally occuring under the same laboratory conditions and in the same strain of animals employed in the test) is desirable for assessing the significance of changes observed in exposed animals.

C. Dose levels and dose selection

1. For risk assessment purposes, at least three dose levels should be used, in addition to the concurrent control group. Dose levels should be spaced to produce a gradation of effects.

2. The highest dose level in rodents should elicit signs of toxicity without substantially altering the normal life span due to effects other than tumors.

3. The lowest dose level should produce no evidence of toxicity. Where there is a usable estimation of human exposure, the lowest dose level should exceed this even though this dose level may result in some signs of toxicity.

4. Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used the dose levels should be spaced to produce a gradation of toxic effects.

5. For rodents, the incidence of fatalities in low and intermediate dose groups and in the controls should be low to permit a meaningful evaluation of the results.

6. For chronic toxicological assessment, a high dose treated satellite and a concurrent control satellite group should be included in the study design. The highest dose for satellite animals should be chosen so as to produce frank toxicity, but not excessive lethality, in order to elucidate a chronic toxicological profile of the test substance. If more than one dose level is selected for satellite dose groups, the doses should be spaced to produce a gradation of toxic effects.

D. Exposure conditions

The animals are dosed with the test substance ideally on a seven-day per week basis over a period of at least 24 months for rats, and 18 months for mice and hamsters, except for the animals in the satellite groups which should be dosed for 12 months.

E. Observation period

It is necessary that the duration of the oncogenicity test comprise the majority of the normal life span of the animals to be used. It has been suggested that the duration of the study should be for the entire lifetime of all animals. However, a few animals may greatly exceed the average lifetime and the duration of the study may be unnecessarily extended and complicate the conduct and evaluation of the study. Rather, a finite period covering the majority of the expected life span of the strain is preferred since the probability is high that, for the great majority of chemicals, induced tumors will occur within such an observation period. The following guidelines are recommended:

1. Generally, the termination of the study should be at 18 months for mice and hamsters and 24 months for rats; however, for certain strains of animals with greater longevity and/or low spontaneous tumor rate, termination should be at 24 months for mice and hamsters and at 30 months for rats. For longer time periods, and where any other species are used, consultation with the Agency in regard to duration of the test is advised.

2. However, termination of the study is acceptable when the number of survivors of the lower doses or control group reach 25 percent. In the case where only the high dose group dies prematurely for obvious reasons of toxicity, this should not trigger termination of the study.

3. The satellite groups and the concurrent satellite control group should be retained in the study for at least 12 months. These groups should be scheduled for sacrifice for an estimation of test-substance-related pathology uncomplicated by geriatric changes.

F. Administration of the test substance

The three main routes of administration are oral, dermal, and inhalation. The choice of the route of administration depends upon the physical and chemical characteristics of the test substance and the form typifying exposure in humans.

1. Oral studies

   a. The animals should receive the test substance in their diet, dissolved in drinking water, or given by gavage or capsule for a period of at least 24 months for rats and 18 months for mice and hamsters.

   b. If the test substance is administered in the drinking water, or mixed in the diet, exposure is continuous.

   c. For a diet mixture, the highest concentration should not exceed five percent.

2. Dermal studies

a. The animals are treated by topical application with the test substance, ideally for at least six hours per day.

b. Fur should be clipped from the dorsal area of the trunk of the test animals. Care should be taken to avoid abrading the skin which could alter its permeability.

c. The test substance should be applied uniformly over a shaved area which is approximately ten percent of the total body surface area. With highly toxic substances, the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

d. During the exposure period, the test substance may be held, if necessary, in contact with the skin with a porous gauze dressing and non-irritating tape. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance.

3. Inhalation studies

a. The animals should be tested with inhalation equipment designed to sustain a dynamic air flow of 12 to 15 air changes per hour, ensure an adequate oxygen content of 19 percent and an evenly distributed exposure atmosphere. Where a chamber is used, its design should minimize crowding of the test animals and maximize their exposure to the test substance. This is best accomplished by individual caging. As a general rule, to ensure stability of a chamber atmosphere, the total "volume" of the test animals should not exceed five percent of the volume of the test chamber. Alternatively, oro-nasal, head-only, or whole body individual chamber exposure may be used.

b. The temperature at which the test is performed should be maintained at 22°C (± 2°). Ideally, the relative humidity should be maintained between 40 to 60 percent, but in certain instances (e.g., tests of aerosols, use of water vehicle) this may not be practicable.

c. Food and water should be withheld during each daily six-hour exposure period.

d. A dynamic inhalation system with a suitable analytical concentration control system should be used. The rate of air flow should be adjusted to ensure that conditions throughout the equipment are essentially the same. Maintenance of slight negative pressure inside the chamber will prevent leakage of the test substance into the surrounding areas.

G. Observation of animals

1. Each animal should be handled and its physical condition appraised at least once each day.

2. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

3. Clinical signs and mortality should be recorded for all animals. Special attention should be paid to tumor development. The time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded.

4. Body weights should be recorded individually for all animals once a week during the first 13 weeks of the test period and at least once every four weeks thereafter, unless signs of clinical toxicity suggest more frequent weighings to facilitate monitoring of health status.

5. When the test substance is administered in the food or drinking water, measurements of food or water consumption, respectively, should be determined weekly during the first 13 weeks of the study and then at approximately monthly intervals unless health status or body weight changes dictate otherwise.

6. At the end of the study period all survivors are sacrificed. Moribund animals should be removed and sacrificed when noticed.

H. Physical measurements

For inhalation studies, measurements or monitoring should be made of the following:

1. The rate of air flow should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

2. During each exposure period the actual concentrations of the test substance should be held as constant as practicable, monitored continuously and measured at least three times during the test period: at the beginning, at an intermediate time and at the end of the period.

3. During the development of the generating system, particle size analysis should be performed to establish the stability of aerosol concentrations. During exposure, analyses should be conducted as often as necessary to determine the consistency of particle size distribution and homogeneity of the exposure stream.

4. Temperature and humidity should be monitored continuously, but should be recorded at intervals of at least once every 30 minutes.

I. Clinical examinations

1. The following examinations should be made on at least 20 rodents of each sex per dose level:

a. Certain hematology determinations (e.g., hemoglobin content, packed cell volume, total red blood cells, total white blood cells, platelets, or other measures of clotting potential) should be performed at termination and should be performed at three months, six months and at approximately six-month intervals thereafter (for those groups on test for longer than 12 months) on blood samples collected from 20 rodents per sex of all groups. These collections should be from the same animals at each interval. If clinical observations suggest a deterioration in health of the animals during the study, a differential blood count of the affected animals should be performed. A differential blood count should be performed on samples from those animals in the highest dosage group and the controls. Differential blood counts should be performed for the next lower group(s) if there is a major discrepancy between the highest group and the controls. If hematological effects were noted in the subchronic test, hematological testing should be performed at 3, 6, 12, 18 and 24 months for a two year study.

b. Certain clinical biochemistry determinations on blood should be carried out at least three times during the test period: just prior to initiation of dosing (baseline data), near the middle and at the end of the test period. Blood samples should be drawn for clinical measurements from at least ten rodents per sex of all groups; if possible, from the same rodents at each time interval. Test areas which are considered appropriate to all studies: electrolyte balance, carbohydrate metabolism and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity. Suggested chemical determinations: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum glutamic-pyruvic transaminase*, serum glutamic oxaloacetic transaminase**, ornithine decarboxylase, gamma glutamyl transpeptidase, blood urea nitrogen, albumen, creatinine phosphokinase, total cholesterol, total bilirubin and total serum protein measurements. Other determinations which may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid/base balance, methemoglobin and cholinesterase activity. Additional clinical biochemistry may be employed where necessary to extend the investigation of observed effects.

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* Now known as serum alanine aminotransferase.

** Now known as serum aspartate aminotransferase.

2. The following should be performed on at least ten rodents of each sex per dose level:

   a. Urine samples from the same rodents at the same intervals as hematological examination above, should be collected for analysis. The following determinations should be made from either individual animals or on a pooled sample/sex/group for rodents: appearance (volume and specific gravity), protein, glucose, ketones, bilirubin, occult blood (semi-quantitatively) and microscopy of sediment (semi-quantitatively).

   b. Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made prior to the administration of the test substance and at the termination of the study. If changes in the eyes are detected all animals should be examined.

J. Gross necropsy

1. A complete gross examination should be performed on all animals, including those which died during the experiment or were killed in moribund conditions.

2. The liver, kidneys, adrenals, brain and gonads should be weighed wet, as soon as possible after dissection to avoid drying. For these organs, at least ten rodents per sex per group should be weighed.

3. The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: all gross lesions and tumors; brain - including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; lungs; trachea; heart; sternum and/or femur with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph nodes; pancreas; gonads; uterus; accessory genital organs (epididymis, prostate, and, if present, seminal vesicles); female mammary gland; aorta; gall bladder (if present); skin; musculature; peripheral nerve; spinal cord at three levels - cervical, midthoracic, and lumbar; and eyes. In inhalation studies, the entire respiratory tract, including nose, pharynx, larynx and paranasal sinuses should be examined and preserved. In dermal studies, skin from sites of skin painting should be examined and preserved.

4. Inflation of lungs and urinary bladder with a fixative is the optimal method for preservation of these tissues. The proper inflation and fixation of the lungs in inhalation studies is considered essential for appropriate and valid histopathological examination.

5. If other clinical examinations are carried out, the information obtained from these procedures should be available before microscopic examination, since they may provide significant guidance to the pathologist.

K. Histopathology

1. The following histopathology should be performed:

   a. Full histopathology on the organs and tissues, listed above, of all non-rodents, of all rodents that died or where killed during the study.

b. All gross lesions in all animals.

c. Target organs in all animals.

d. Lungs, liver and kidneys of all animals. Special attention to examination of the lungs of rodents should be made for evidence of infection since this provides an assessment of the state of health of the animals.

2. If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

3. In case the results of the experiment give evidence of substantial alteration of the animals' normal longevity or the induction of effects that might affect a toxic response, the next lower dose level should be examined as described above.

4. An attempt should be made to correlate gross observations with microscopic findings.

III. DATA AND REPORTING

A. Treatment of results

1. Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

2. All observed results, quantitative and incidental, should be evaluated by an appropriate statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

B. Evaluation of study results

1. The findings of a combined chronic toxicity/oncogenicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects, the necropsy and histopathological findings. The evaluation will include the relationship between the dose of the test

substance and the presence, incidence and severity of abnormalities (including behavioral and clinical abnormalities), gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects.

2. In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

3. In order for a negative test to be acceptable, it should meet the following criteria: no more than ten percent of any group is lost due to autolysis, cannibalism, or management problems; and survival in each group is no less than 50 percent at 18 months for mice and hamsters and at 24 months for rats.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Group animal data

   Tabulation of toxic response data by species, strain, sex and exposure level for:

   a. Number of animals dying;

   b. Number of animals showing signs of toxicity; and

   c. Number of animals exposed.

2. Individual animal data

   a. Time of death during the study or whether animals survived to termination;

   b. Time of observation of each abnormal sign and its subsequent course;

   c. Body weight data;

   d. Food and water consumption data, when collected;

e. Results of ophthalmological examination, when performed;

f. Hematological tests employed and all results;

g. Clinical biochemistry tests employed and all results;

h. Necropsy findings;

i. Detailed description of all histopathological findings;

j. Statistical treatment of results where appropriate; and

k. Historical control data, if taken into account.

In addition, for inhalation studies the following should be reported:

3. Test conditions

a. Description of exposure apparatus including design, type, dimensions, source of air, system for generating particulates and aerosols, method of conditioning air, treatment of exhaust air and the method of housing the animals in a test chamber.

b. The equipment for measuring temperature, humidity, and particulate aerosol concentrations and size should be described.

4. Exposure data

These should be tabulated and presented with mean values and a measure of variability (e.g. standard deviation) and should include:

a. Airflow rates through the inhalation equipment;

b. Temperature and humidity of air;

c. Nominal concentration (total amount of test substance fed into the inhalation equipment divided by volume of air);

d. Actual concentration in test breathing zone; and

e. Particle size distribution (e.g. median aerodynamic diameter of particles with standard deviation from the mean).

IV. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

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5. Food and Drug Administration Advisory Committee on Protocols for Safety Evaluation: Panel on Carcinogenesis. 1971. Report on Cancer Testing in the Safety of Food Additives and Pesticides. Toxicology and Applied Pharmacology. 20:419-438.

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HG-Organ/Tissue-Dermal Irrit
1983

PRIMARY DERMAL IRRITATION

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I. PURPOSE

In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and/or corrosive effects on skin of mammals is an important initial step. Information derived from this test serves to indicate the existence of possible hazards likely to arise from exposure of the skin to the test substance.

II. DEFINITIONS

A. Dermal irritation is the production of reversible inflammatory changes in the skin following the application of a test substance.

B. Dermal corrosion is the production of irreversible tissue damage in the skin following the application of the test substance.

III. PRINCIPLE OF THE TEST METHOD

A. The substance to be tested is applied in a single dose to the skin of several experimental animals, each animal serving as its own control. The degree of irritation is read and scored at specified intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to permit a full evaluation of the reversibility or irreversibility of the effects observed but need not exceed 14 days.

B. When testing solids (which may be pulverized if considered necessary), the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle, to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

C. Strongly acidic or alkaline substances, for example with a demonstrated pH of 2 or less, or 11.5 or greater, need not be tested for primary dermal irritation, owing to their predictable corrosive properties.

D. The testing of materials which have been shown to be highly toxic by the dermal route is unnecessary.

IV. TEST PROCEDURES

A. Animal selection

1. Species and strain

The albino rabbit is recommended as the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection.

2. Number of animals

At least 6 healthy adult animals should be used unless, justification/reasoning for using fewer animals is provided.

B. Control animals

Separate animals are not recommened for an untreated control group. Adjacent areas of untreated skin of each animal may serve as a control for the test.

C. Dose level

A dose of 0.5 ml of liquid or 500 mg of solid or semi-solid is applied to the test site.

D. Preparation of animals' skins

Approximately 24 hours before the test, fur should be removed from the test area by clipping or shaving from the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin. Only animals with healthy intact skin should be used.

E. Application of the test substance

1. The recommended exposure duration is 4-hours. Longer exposure may be indicated under certain conditions (e.g. expected pattern of human use and exposure). At the end of the exposure period, residual test substance should generally be removed, where practicable, using water or an appropriate solvent, without altering the existing response or the integrity of the epidermis.

2. The test substance should be applied to a small area (approximately 6 cm2) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In the case of liquids or some pastes, it may be necessary to apply the test substance to the gauze patch and then apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable semi-occlusive dressing for the duration of the exposure period. However, the use of an occlusive dressing may be considered appropriate in some cases. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.

F. Observation period

The duration of the observation period should be at least 72 hours, but should not be rigidly fixed. It should be sufficient to fully evaluate the reversibility or irreversiblity of the effects observed. It need not exceed 14 days after application.

G. Clinical examination and scoring

After removal of the patch, animals should be examined for signs of erythema and edema and the responses scored within 30-60 minutes, and then at 24, 48 and 72 hours after patch removal.

Dermal irritation should be scored and recorded according to the grades in Table 1, below. Further observations may be needed, as necessary, to establish reversibility. In addition to the observation of irritation, any lesions and other toxic effects should be fully described.

Table 1: Evaluation of Skin Reaction

Erythema and Eschar Formation	Value
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Maximum possible	4

Edema Formation	Value
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 millimeter)	3
Severe edema (raised more than 1 millimeter and extending beyond area of exposure	4
Maximum possible.	4

V. DATA AND REPORTING

A. Data should be summarized in tabular form, showing for each individual animal the irritation scores for erythema and edema at 30 to 60 minutes, 24, 48 and 72 hours after patch removal, any lesions, a description of the degree and nature of irritation, corrosion or reversibility, and any other toxic effects observed.

B. Evaluation of results

The dermal irritation scores should be evaluated in conjunction with the nature and reversibility or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material. They should be viewed as reference values which are only meaningful when supported by a full description and evaluation of the observations. The use of an occlusive dressing is a severe test and the results are relevant to very few likely human exposure conditions.

C. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Physical nature and, where appropriate, concentration, and pH value for the test substance;
2. Species and strain;
3. Tabulation of irritation response data for each individual animal for each observation time period (e.g. 30 to 60 minutes, 24, 48, 72 hours after patch removal);
4. Description of any lesions observed;
5. Narrative description of the degree and nature of irritation observed; and
6. Description of any toxic effects other than dermal irritation.

VI. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Draize, J.H. 1959. Third Printing: 1975. "Dermal Toxicity," in "Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics." Association of Food and Drug Officials of the United States. PP. 46-59.

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HG-Organ/Tissue-Repro/Fert
October, 1983

REPRODUCTION AND FERTILITY EFFECTS

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I. PURPOSE

This guideline for two-generation reproduction testing is designed to provide general information concerning the effects of a test substance on gonadal function, conception, parturition, and the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, and preliminary data on teratogenesis and serve as a guide for subsequent tests.

II. PRINCIPLE OF THE TEST METHOD

The test substance is administered to parental (P) animals prior to their mating, during the resultant pregnancies, and through the weaning of their F_1 offspring. The substance is then administered to selected F_1 offspring during their growth into adulthood, mating, and production of an F_2 generation, up until the F_2 generation is weaned.

III. TEST PROCEDURES

A. Animal selection

1. Species and strain

The rat is the preferred species. If another mammalian species is used, the tester should provide justification/reasoning for its selection. Strains with low fecundity should not be used.

2. Age

Parental (P) animals should be about 5-8 weeks old at the start of dosing.

3. Sex

a. For an adequate assessment of fertility, both males and females should be studied.

b. The females should be nulliparous and non-pregnant.

4. Number of animals

Each test and control group should contain at least 20 males and a sufficient number of females to yield at least 20 pregnant females at or near term.

B. Control groups

1. A concurrent control group should be used. This group should be an untreated or sham treated control group or if a vehicle is used in administering the test substance, a vehicle control group.

2. If a vehicle is used in administering the test substance, the control group should receive the vehicle in the highest volume used.

3. If a vehicle or other additive is used to facilitate dosing, it should not interfere with absorption of the test substance or produce toxic effects.

C. Dose levels and dose selection

1. At least three dose levels and a concurrent control should be used.

2. The highest dose level should induce toxicity but not high levels of mortality in the parental (P) animals.

3. The lowest dose level should not produce any grossly observable evidence of toxicity.

4. Ideally the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, dose levels should be spaced to produce a gradation of toxic effects.

D. Exposure conditions

The animals should be dosed with the test substance, ideally, on a seven-days per week basis.

1. Dosing, mating, delivery, and sacrifice schedule.

a. Daily dosing of the parental (P) males and females should begin when they are 5-8 weeks old. For both sexes, dosing should be continued for at least ten weeks before the mating period.

b. Dosing of P males should continue through the three week mating period. At the end of the mating period, P males may be sacrificed and examined, or may be retained for possible production of second litter. It these animals are retained for a second litter, dosing should be continued. Dosing of the F_1 males saved for mating should continue from the time they are weaned through the period they are mated with the F_1 females (11 weeks). F_1 males may be sacrificed after the F_1 mating period.

c. Daily dosing of the P females should continue through the three week mating period, pregnancy, and to the weaning of the F_1 offspring. Dosing of the F_1 females saved for mating should continue from the time they are weaned, through the period they are mated with the F_1 males (11 weeks from time of weaning) pregnancy, and to the weaning of the F_2 offspring.

2. All animals are sacrificed as scheduled.

a. All P males should be sacrificed at the end of the three week mating period, or may be retained for possible production of a second litter. If these animals are retained for a second litter, dosing should be continued.

b. F_1 males selected for mating should be sacrificed at the end of the three week period of the F_1 mating.

c. F_1 males and females not selected for mating should be sacrificed when weaned.

d. The P females should be sacrificed upon weaning of their F_1 offspring.

e. F_1 dams and their F_2 offspring are sacrificed when the offspring are weaned.

E. Administration of the test substance

1. Oral studies

a. It is recommended that the test substance be administered in the diet or drinking water.

b. If administered by gavage or capsule, the dosage administered to each animal prior to mating should be based on the individual animal's body weight and adjusted weekly. During pregnancy the dosage should be based on the body weight at Day 0 and 6 of pregnancy.

2. If another route of administration is used, the tester should provide justification and reasoning for its selection.

F. Mating procedure

1. Parental

a. For each mating, each female should be placed with a single male from the same dose level until pregnancy occurs or one week has elapsed. If mating has not ocurred after one week, the female should be placed with a different male. Paired matings should be clearly identified.

b. Those pairs that fail to mate should be evaluated to determine the cause of the apparent infertility. This may involve such procedures as additional opportunities to mate with proven fertile males or females, histological examination of the reproductive organs, and examination of the estrus or spermatogenic cycles.

c. Each day, the females should be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day vaginal plugs or sperm are found.

2. F_1 cross

   a. For mating the F_1 offspring, one male and one female are randomly selected at weaning from each litter for cross mating with another pup of the same dose level but different litter, to produce the F_2 generation.

   b. F_1 males and females not selected for mating are sacrificed upon weaning.

3. Special housing

   After evidence of copulation, pregnant animals should be caged separately in delivery or maternity cages and provided with nesting materials.

4. Standardization of litter sizes

   a. On day 4 after birth, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, 4 males and 4 females per litter.

   b. Whenever the number of male or female pups prevents having 4 of each sex per litter, partial adjustment (for example, 5 males and 3 females) is permitted. Adjustments are not appropriate for litters of less than 8 pups.

   c. Elimination of runts only is not appropriate.

   d. Adjustments of the F_2 litters is conducted in the same manner.

G. Observation of animals

1. A gross examination should be made at least once each day. Pertinent behavioral changes, signs of difficult or prolonged parturition, and all signs of toxicity, including mortality, should be recorded. These observations should be reported for each individual animal. Food consumption for all animals should be monitored weekly.

2. The duration of gestation should be calculated from Day 0 of pregnancy.

3. Each litter should be examined as soon as possible after delivery for the number of pups, stillbirths, live births, sex, and the presence of gross anomalies. Dead pups and pups sacrificed at day 4 should be preserved and studied for possible defects. Live pups should be counted and litters weighed, by weighing each individual pup at birth, or soon thereafter, and on days 4, 7, 14 and 21 after parturition.

4. Physical or behavioral abnormalities observed in the dams or offspring should be recorded.

5. P males and females should be weighed on the first day of dosing and weekly thereafter. F_1 litters should be weighed at birth, or soon thereafter, and on days 4, 7, 14 and 21. In all cases, litter weights should be calculated from the weights of the individual pups.

H. Gross necropsy

1. A complete gross examination should be performed on all animals, including those which died during the experiment or were killed in moribund conditions.

2. Special attention should be directed to the organs of the reproductive system.

3. The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination: vagina; uterus; ovaries; testes; epididymides; seminal vesicles; prostate, pituitary gland; and, target organ(s) of all P and F_1 animals selected for mating.

I. Histopathology

1. The following histopathology should be performed:

   a. Full histopathology on the organs listed above for all high dose, and control P_1 and F_1 animals selected for mating.

   b. Organs demonstrating pathology in these animals should then be examined in animals from the other dose groups.

c. Microscopic examination should be made of all tissues showing gross pathological changes.

IV. DATA AND REPORTING

A. Treatment of Results

Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals pregnant, the types of change and the precentage of animals displaying each type of change.

B. Evaluation of study results

1. An evaluation of test results, including the statistical analysis, based on the clinical findings, the gross necropsy findings, and the microscopic results, should be made and supplied. This should include an evaluation of the relationship, or lack thereof, between the animals' exposure to the test substance and the incidence and severity of all abnormalities.

2. In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Toxic response data by sex and dose, including fertility, gestation, viability and lactation indices, and length of gestation;

2. Species and strain;

3. Time of death during the study or whether animals survived to termination;

4. Toxic or other effects on reproduction, offspring, or postnatal growth;

5. Time of observation of each abnormal sign and its subsequent course;
6. Body weight data for P, F_1, and F_2 animals;
7. Necropsy findings;
8. Detailed description of all histopathological findings; and
9. Statistical treatment of results where appropriate.

V. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Clermont, Y., Perry, B. 1957. Quantitative Study of the Cell Population of the Seminiferous Tubules in Immature Rats. American Journal of Anatomy. 100:241-267.

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HG-Organ/Tissue-Terato
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TERATOGENICITY STUDY

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I. PURPOSE

The teratogenicity study is designed to determine the potential of the test substance to induce structural and/or other abnormalities in the fetus which may arise from exposure of the mother during pregnancy.

II. DEFINITIONS

A. Teratogenicity is the property of a chemical that causes structural or functional abnormalities during the period of embryonic development.

III. PRINCIPLE OF THE TEST METHOD

The test substance is administered in graduated doses, for at least that part of the pregnancy covering the period of organogenesis, to several groups of pregnant experimental animals, one dose level being used per group. Shortly before the expected date of delivery, the pregnant females are sacrificed, the uteri removed, and the contents examined for embryonic or fetal deaths, and live fetuses.

IV. LIMIT TEST

If a test at a dose of at least 1000 mg/kg body weight, using the procedures described for this study, produces no observable embryo toxicity or teratogenicity, then a full study using three dose levels might not be necessary.

V. TEST PROCEDURES

A. Animal selection

1. Species and strain

Testing should be performed in at least 2 mammalian species. The preferred species are the rat and the rabbit. If other mammalian species are used, the tester should provide justification/reasoning for their selection. Commonly used laboratory strains should be employed. The strain should not have low fecundity and should preferably be characterized for its sensitivity to teratogens.

2. Age

Young adult animals (nulliparous females) should be used.

3. Sex

Pregnant female animals should be used at each dose level.

4. Number of animals

At least 20 pregnant rats, mice or hamsters or 12 pregnant rabbits are recommended at each dose level. The objective is to ensure that sufficient pups are produced to permit meaningful evaluation of the teratogenic potential of the test substance.

B. Control group

A concurrent control group should be used. This group should be an untreated or sham treated control group, or, if a vehicle is used in administering the test substance, a vehicle control group. Except for treatment with the test substance, animals in the control group(s) should be handled in an identical manner to test group animals.

C. Dose levels and dose selection

1. At least 3 dose levels with a control and, where appropriate, a vehicle control, should be used.

2. The vehicle should neither be teratogenic nor have effects on reproduction.

3. To select the appropriate dose levels, a pilot or trial study may be advisable. It is not always necessary to carry out a trial study in pregnant animals. Comparison of the results from a trial study in non-pregnant, and the main study in pregnant animals will demonstrate if the test substance is more toxic in pregnant animals. If a trial study is carried out in pregnant animals, the dose producing embryonic or fetal lethalities or maternal toxicity should be determined.

4. Unless limited by the physical/chemical nature or biological properties of the substance, the highest dosage level should induce some overt maternal toxicity such as slight weight loss, but not more than 10 percent maternal deaths.

5. The lowest dose level should not produce any grossly observable evidence of toxicity.

6. Ideally, the intermediate dose level(s) should produce minimal observable toxic effects. If more than one intermediate dose is used, the dose levels should be spaced to produce a gradation of toxic effects.

D. Observation period

Day 0 in the test is the day on which a vaginal plug and/or sperm are observed. The dose period should cover the period of major organogenesis. This may be taken as days 6-15 for rat and mouse, 6-14 for hamster, or 6-18 for rabbit.

E. Administration of test substance

The test substance or vehicle is usually administered orally, by oral intubation unless the chemical or physical characteristics of the test substance or pattern of human exposure suggest a more appropriate route of administration. The test substance should be administered at the same time each day.

F. Exposure conditions

The female test animals are treated with the test substance daily throughout the appropriate treatment period. When given by gavage, the dose may be based on the weight of the females at the start of substance administration, or, alternatively, in view of the rapid weight gain which takes place during pregnancy, the animals may be weighed periodically and the dosage based on the most recent weight determination.

G. Observation of animals

1. A gross examination should be made at least once each day.

2. Additional observations should be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals).

3. Signs of toxicity should be recorded as they are observed, including the time of onset, the degree and duration.

4. During the treatment and observation periods, cage-side observations should include, but not be limited to: changes in skin and fur, eye and mucous membranes, as well as respiratory, autonomic and central nervous systems, somatomotor activity and behavioral pattern.

5. Measurements should be made weekly of food consumption for all animals in the study.

6. Animals should be weighed at least weekly.

7. Females showing signs of abortion or premature delivery should be sacrificed and subjected to a thorough macroscopic examination.

H. Gross necropsy

1. At the time of sacrifice or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy.

2. Immediately after sacrifice or death, the uterus should be removed and the contents examined for embryonic or fetal deaths and the number of viable fetuses. The degree of resorption should be described in order to help estimate the relative time of death.

3. The number of corpora lutea should be determined for all species except mice.

4. The sex of the fetuses should be determined and they should be weighed individually, the weights recorded, and the mean fetal weight derived.

5. Following removal, each fetus should be examined externally.

6. For rats, mice and hamsters, one-third to one-half of each litter should be prepared and examined for skeletal anomalies, and the remaining part of each litter should be prepared and examined for soft tissue anomalies using appropriate methods.

7. For rabbits, each fetus should be examined by careful dissection for visceral anomalies and then examined for skeletal anomalies.

VI. DATA AND REPORTING

A. Treatment of results

Data should be summarized in tabular form, showing for each test group; the number of animals at the start of the test, the number of pregnant animals, the number and percentages of live fetuses and the number of fetuses with any soft tissue or skeletal abnormalities.

B. Evaluation of results

The findings of a teratogenicity study should be evaluated in terms of the observed effects and the dose levels producing effects. It is necessary to consider the historical teratogenicity data on the species/strain tested. A properly conducted teratogenicity study should provide a satisfactory estimation of a no-effect level.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Toxic response data by dose;
2. Species and strain;
3. Time of death during the study or whether animals survived to termination;
4. Time of onset and duration of each abnormal sign and its subsequent course;
5. Food and body weight data;

6. Pregnancy and litter data; and

7. Fetal data (live/dead, sex, soft tissue and skeletal defects, resorptions).

VII. REFERENCES

The following references may be helpful in developing acceptable procotols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. DHEW. 1975. Department of Health and Welfare. The Testing of Chemicals for Carcinogenicity, Mutagenicity and Teratogenicity. Canada: The Honorable Marc Lalonde, Minister of Health and Welfare, Department of Health and Welfare. 183 pp.

2. NAS. 1977. National Academy of Sciences. Principles and Procedures for Evaluating the Toxicity of Household Substances. Washington, D.C.: A report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences. 130 pp.

3. WHO. 1967. World Health Organization. Principles for the Testing of Drugs for Teratogenicity. WHO Technical Report Series No. 364. Geneva: World Health Organization. 18 pp.

THE *SALMONELLA TYPHIMURIUM* REVERSE MUTATION ASSAY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

The *Salmonella typhimurium* histidine (his) reversion system is a microbial assay which measures $\text{his}^- \longrightarrow \text{his}^+$ reversion induced by chemicals which cause base changes or frameshift mutations in the genome of this organism.

II. DEFINITIONS

A. A reverse mutation assay in *Salmonella typhimurium* detects mutation in a gene of a histidine requiring strain to produce a histidine independent strain of this organism.

B. Base pair mutagens are agents which cause a base change in the DNA. In a reversion assay, this change may occur at the site of the original mutation or at a second site in the chromosome.

C. Frameshift mutagens are agents which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, sodium azide, 2-nitrofluorene, 9-aminoacridine or 2-aminoanthracene.

IV. TEST METHOD

A. Principle

Bacteria are exposed to test chemical with and without a metabolic activation system and plated onto minimal medium. After a suitable period of incubation, revertant colonies are counted and compared to the number of spontaneous revertants in an untreated and/or vehicle control culture.

B. Description

Several methods for performing the test have been described. Among those used are:

1. the direct plate incorporation method,

2. the preincubation method,
3. the suspension method,
4. the gradient plate method, and
5. the desiccator method.

The procedure described here is for the direct plate incorporation method.

C. Strain selection

1. Designation

At the present time four strains, TA 1535, TA 1537, TA 98 and TA 100 should be used. The use of strain TA 1538 is left to the discretion of the investigator. Other strains may be utilized when appropriate.

2. Preparation and storage

Recognized methods of stock culture preparation and storage should be used. The requirement of histidine for growth should be demonstrated for each strain. Other phenotypic characteristics should be checked using such methods as crystal violet sensitivity and resistance to ampicillin. Spontaneous reversion frequency should be in the range expected either as reported in the literature or as established in the laboratory by historical control values.

3. Bacterial growth

Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^8-10^9 cells per ml).

D. Metabolic activation

Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor supplemented postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents. The use of other species, tissues or techniques may also be appropriate.

E. Control groups

1. Concurrent controls

Concurrent positive and negative (untreated and/or vehicle) controls should be included in each experiment. Positive controls should insure both strain responsiveness and efficacy of the metabolic activation system.

2. Strain specific positive controls

Strain specific positive controls should be included in the assay. Examples of strain specific positive controls are as follows:

a. Strain TA 1535, TA 100, sodium azide;
b. TA 98, 2-nitrofluorene;
c. TA 1537, 9-aminoacridine.

3. Positive controls to ensure the efficacy of the activation system

The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test. 2-Aminoanthracene is an example of a positive control compound in tests using postmitochondrial fractions from the livers of rodents treated with enzyme inducing agents such as Aroclor-1254.

4. Other positive controls

Other positive control reference substances may be used.

F. Test chemicals

1. Vehicle

Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

2. Exposure concentrations

a. The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. Toxicity may be evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn or by the degree of survival of treated cultures. Relatively insoluble compounds should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

b. Generally, a maximum of 5 mg/plate for pure substances is considered acceptable. At least 5 different amounts of test substance should be tested with adequate intervals between test points.

c. When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations.

V. TEST PERFORMANCE

A. Direct plate incorporation method

For this test without metabolic activation, test chemical and 0.1 ml of a fresh bacterial culture should be added to 2.0 ml of overlay agar. For tests with metabolic activation, 0.5 ml of activation mixture containing an adequate amount of postmitochondrial fraction should be added to the agar overlay after the addition of test chemical and bacteria. Contents of each tube should be mixed and poured over the surface of a selective agar plate. Overlay agar should be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate should be counted.

B. Other methods

Other methods may also be appropriate.

C. Media

An appropriate selective medium with an adequate overlay agar should be used.

D. Incubation conditions

All plates within a given experiment should be incubated for the same time period. This incubation period should be for 48-72 hours at 37 C.

E. Number of cultures

In general, all plating should be done at least in triplicate. If scientifically justified, the use of duplicates may be acceptable. All results should be confirmed in an independent experiment.

VI. DATA AND REPORT

A. Treatment of results

Data should be presented as number of revertant colonies per plate for each replicate and dose. The numbers of revertant colonies on both negative (untreated and/or vehicle) and positive control plates should also be presented. Individual plate counts, the mean number of revertant colonies per plate and standard deviation should be presented for test chemical and positive and negative (untreated and/or vehicle) controls.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of revertants. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of revertants or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results from the *S. typhimurium* reverse mutation assay indicate that, under the test conditions, the test substance induces point mutations by base changes or frameshifts in the genome of this organism.

2. Negative results indicate that under the test conditions the test substance is not mutagenic in *S. typhimurium*.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. bacterial strain used;

2. details of both the protocol used to prepare metabolic activation system and its use in the assay;

3. dose levels and rationale for selection of dose;

4. positive and negative controls;

5. individual plate counts, mean number of revertant colonies per plate, standard deviation;

6. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

2. de Serres FJ, Shelby MD. 1979. The *Salmonella* mutagenicity assay: recommendations. Science 203:563-565.

3. McMahon RE, Clive JC, Thompson CZ. 1979. Assay of 855 test chemicals in ten tester strains using a new modification of the Ames test for bacterial mutagens. Cancer Res 39:682-693.

4. Thompson ED, Melampy PJ. 1981. An examination of the quantitative suspension assay for mutagenesis with strains of *Salmonella typhimurium*. Environmental Mutagenesis 3:453-465.

5. Vogel HJ, Bonner DM. 1956. Acetylornithinase of *E. coli*: partial purification and some properties. J Biol Chem 218:97-106.

HG-Gene Muta-E. coli
October, 1983

THE *ESCHERICHIA COLI* WP2 AND WP2 *uvrA* REVERSE MUTATION ASSAYS

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U. S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

The *E. coli* tryptophan (*trp*) reversion system is a microbial assay which measures $trp^- \rightarrow trp^+$ reversion induced by chemicals which cause mutations in the genome of this organism.

II. DEFINITION

A reverse mutation assay in *E. coli* detects mutation in a gene of a tryptophan requiring strain to produce a tryptophan independent strain of this organism.

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, 4-nitroquinoline oxide, methyl methanesulfonate, or 2-aminoanthracene.

IV. TEST METHOD

A. Principle

Bacteria are exposed to test chemical with and without metabolic activation and plated onto minimal medium. After a suitable period of incubation, revertant colonies are counted and compared to the number of spontaneous revertants in an untreated and/or vehicle control culture.

B. Description

Several methods for performing the test have been described. Among those used are:

1. the direct plate incorporation method,
2. the preincubation method,
3. the treat and plate method,
4. the modified fluctuation test, and
5. the desiccator method.

The procedure described here is for the direct plate incorporation method.

C. Strain selection

1. Designation

At the present time, three strains, WP2, WP2 uvrA and WP2 uvrA/pKM101 should be used. Other strains may be utilized when appropriate.

2. Preparation and storage

Recognized methods of stock culture preparation and storage should be used. The requirement of tryptophan for growth should be demonstrated for each strain. Other phenotypic characteristics should be checked using such methods as sensitivity to mitomycin C and resistance to ampicillin. Spontaneous reversion frequency should be in the range expected either as reported in the literature or as established in the laboratory by historical control values.

3. Bacterial growth

Fresh cultures of bacteria should be grown up to the late exponential or early stationary phase of growth (approximately 10^8-10^9 cells per ml).

D. Metabolic activation

Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor supplemented postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents. The use of other species, tissues or techniques may also be appropriate.

E. Control groups

1. Concurrent controls

Concurrent positive and negative (untreated and/or vehicle) controls should be included in each experiment.

2. Direct acting positive controls

Examples of positive controls for assays performed without metabolic activation include methyl methanesulfonate and 4-nitroquinoline oxide.

3. Positive controls to ensure the efficacy of the activation system

The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test. 2-aminoanthracene is an example of a positive control compound in tests using postmitochondrial fractions from the livers of rodents treated with enzyme inducing agents such as Aroclor-1254.

4. Other positive controls

Other positive control reference substances may be used.

F. Test chemicals

1. Vehicle

Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

2. Exposure concentrations

a. The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. Toxicity may be evidenced by a reduction in the number of spontaneous revertants, a clearing of the background lawn or by the degree of survival of treated cultures. Relatively insoluble chemicals should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

b. Generally, a maximum of 5 mg/plate for pure substances is considered acceptable. At least 5 different amounts of test substance should be tested with adequate intervals between the test points.

c. When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations.

V. TEST PERFORMANCE

A. Direct plate incorporation method

For this test without metabolic activation, test chemical and 0.1 ml of a fresh bacterial culture should be added to 2.0 ml of overlay agar.

For tests with metabolic activation, 0.5 ml of activation mixture containing an adequate amount of postmitochondrial fraction should be added to the overlay agar after the addition of test chemical and bacteria. Contents of each tube should be mixed and poured over the surface of a selective agar plate. Overlay agar should be allowed to solidify before incubation. At the end of the incubation period, revertant colonies per plate should be counted.

B. Other methods

Other methods may also be appropriate.

C. Media

An appropriate selective medium with an adequate overlay agar should be used.

D. Incubation conditions

All plates in a given experiment should be incubated for the same time period. This incubation period should be for 48-72 hours at 37 C.

E. Number of cultures

All plating should be done at least in triplicate. If scientifically justified, the use of duplicates may be acceptable. All results should be confirmed in an independent experiment.

VI. DATA AND REPORT

A. Treatment of results

Data should be presented as number of revertant colonies per plate for each replicate and dose. The numbers of revertant colonies on both negative (untreated and/or vehicle) and positive control plates should also be presented. Individual plate counts, the mean number of revertant colonies per plate and standard deviation should be presented for test chemical and positive and negative (untreated and/or vehicle) controls.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of revertants. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of revertants or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results from the *E. coli* reverse mutation assay indicate that, under the test conditions, the test substance induces mutations in the genome of this organism.

2. Negative results indicate that under the test conditions the test substance is not mutagenic in *E. coli*.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. bacterial strain used;
2. details of both the protocol used to prepare the metabolic activation system and of its use in the assay,
3. dose levels and rationale for selection of dose;
4. positive and negative controls;
5. individual plate counts, mean number of revertant colonies per plate, standard deviation;
6. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.
2. Brusick DJ, Simmon VF, Rosenkranz HS, Ray VA, Stafford RS. 1980. An evaluation of the *Escherichia coli* WP2 and WP2 *uvrA* reverse mutation assay. Mutation Research 76:169-190.
3. Green MHL, Muriel WJ. 1976. Mutagen testing using trp^+ in *Escherichia coli*. Mutation Research 38:3-32.
4. Vogel HJ, Bonner DM. 1956. Acetylornithinase of *E. coli*: partial purification and some properties. *J Biol Chem* 218:97-106.

HG-Gene Muta-*A. nidulans*
October, 1983

GENE MUTATION IN *ASPERGILLUS NIDULANS*

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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I. PURPOSE

Aspergillus nidulans is a eukaryotic fungus which has been developed to detect and study a variety of genetic phenomena including chemically induced mutagenesis. *A. nidulans* can be used to detect both forward and reverse gene mutation. These mutations are detected by changes in colonial morphology or nutritional requirements in treated populations. The methionine and 2-thioxanthine forward mutation systems can be used to detect mutations in *A. nidulans*.

II. DEFINITION

A forward mutation is a gene mutation from the wild (parent) type to the mutant condition.

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, ethyl methanesulfonate, cyclophosphamide or aflatoxin B_1.

IV. TEST METHOD

A. Principle

Conidia are exposed to test chemical both with and without metabolic activation and plated on selective medium to determine changes in colonial morphology or nutritional requirements. At the end of a suitable incubation period, mutant colonies are counted and compared to the number of spontaneous mutants in an untreated control culture. Simultaneous determination of survival permits calculation of mutation frequency.

B. Description

Tests for mutation in *A. nidulans* are performed in liquid suspension. Treated conidia are plated on selective medium to determine changes in nutritional requirements or colonial morphology.

C. Strain selection

1. Designation

For the methionine and 2-thioxanthine systems the haploid Glascow *biA1; meth G1* strain is the most commonly used strain although other strains may be appropriate. Any translocation-free strain which produces green colonies on thioxanthine free medium and yellow colonies on medium containing thioxanthine may be used in the thioxanthine system.

2. Preparation and storage

Stock culture preparation and storage, growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented.

3. Media

Any medium which supports growth and a characteristic colonial morphology may be used in the assay.

D. Preparation of conidia

Prior to chemical treatment, conidia from 4-5 single colonies of the appropriate strain are grown at 37 C on complete medium. At the end of the incubation period, conidia are collected, conidial chains broken up, mycelial debris removed and conidia concentrated prior to removal of the germination inhibitory substance. Germination inhibitory substance should be removed by Tween 80 or diethyl ether.

E. Metabolic activation

Conidia should be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

F. Control groups

Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation should be included in each experiment.

G. Test chemicals

1. Vehicle

Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

2. Exposure concentrations

a. The test should initially be performed over a broad range of concentrations selected on the basis of a preliminary assay. Effective treatment times should also be selected in the preliminary assay.

b. Each test should include five treatment points, two at fixed concentrations for different time periods, and three at varying concentrations for fixed periods of time.

c. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of a metabolic activation system. Relatively insoluble chemicals should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

d. When appropriate, a positive response should be confirmed by using a narrow range of test concentrations.

V. TEST PERFORMANCE

A. Treatment

Germinating or quiescent conidia in liquid suspension should be exposed to the test chemical at 37 C under conditions of yellow light and controlled pH and oxygen tension. At the end of the exposure period, treatment should be terminated by repeated centrifugation and washing of the conidia or by dilution. Chemical neutralization of the test agent may also be used but is not recommended.

B. Media

1. Methionine system

For the methionine system, condidia should be plated on methionine deficient medium for mutant selection and on medium supplemented with methionine to determine survival.

2. Thioxanthine system

For the 2-thioxanthine system, treated conidia should be plated on nitrogen-free glucose and salts minimal medium containing 2-thioxanthine.

After incubation, green colonies should be counted and isolated by restreaking. The isolated colonies should be classified on the basis of genetic criteria. Yellow, wild-type colonies will grow on the same plate. This permits concurrent determination of survival and an estimation of mutation frequency.

C. Determination of mutation frequency and viability

In both systems, mutation frequency and viability should be determined immediately before and immediately after chemical treatment.

D. Incubation conditions

All incubations should be at 37 C. Incubation time will vary depending upon system and endpoint (mutation or viability) being determined.

E. Number of cultures

1. At least 10 independent plates per concentration with no more than 20 colonies per plate should be used in the methionine system.

2. Fifteen to 20 plates per concentration are preferred for the 2-thioxanthine system.

VI. DATA AND REPORT

A. Treatment of results

Individual plate counts for test substance and controls should be presented for both mutation induction and survival. The mean number of colonies per plate and standard deviation should also be presented. Data should be presented in tabular form indicating, as applicable, numbers of colonies counted, and numbers and classification of mutants identified. Sufficient detail should be provided for verification of survival and mutation frequencies.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of mutant colonies. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of mutant colonies or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaulation

1. Positive results from the methionine and 2-thioxanthine systems in *A. nidulans* indicate that, under the test conditions, the test substance causes gene (point) mutations in the DNA of this organism caused by base pair changes and small deletions in the genome.

2. Negative results indicate that under the test conditions the test chemical is not mutagenic in *A. nidulans*.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. strain of organism used in the assay;
2. test chemical vehicle, doses used and rationale for dose selection, toxicity data;
3. method used for preparation of conidia;
4. treatment conditions, including length of exposure and method used to stop treatment;
5. details of both the protocol used to prepare the metabolic activation system and of its use in the assay;
6. incubation times and temperature;
7. positive and negative controls;
8. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

2. Kafer E, Scott BR, Dorn GL, Stafford RS. 1982. Aspergillus nidulans: systems and results of tests for chemical induction of mitotic segregation and mutation. I. Diploid and duplication assay systems: a report of the U.S. EPA's Gene-Tox Program. Mutation Research 98:1-48.

3. Munson RJ, Goodhead DT. 1977. Relation between induced mutation frequency and cell survival: a theoretical approach and an examination of experimental data for eukaryotes. Mutation Research 42:145-159.

4. Scott BR, Dorn GL, Kafer E, Stafford RS. 1982. Aspergillus nidulans: systems and results of tests for mitotic segregation and mutation. II. Haploid assay systems and overall response of all systems: a report of the U.S. EPA's Gene-Tox Program. Mutation Research 98:49-94.

HG-Gene Muta-*N. crassa*
October, 1983

GENE MUTATION IN *NEUROSPORA CRASSA*

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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I. PURPOSE

Neurospora crassa is a eukaryotic fungus which has been developed to detect and study a variety of genetic phenomena including chemically induced mutagenesis. *N. crassa* can be used to detect both forward and reverse gene mutation. These mutations are detected by biochemical or morphological changes in the treated population. The most commonly used mutation assay in *N. crassa* measures forward mutation in the *ad-3* region of the genome.

II. DEFINITION

A forward mutation is a gene mutation from the wild (parent) type to the mutant condition.

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, ethyl- or methyl methanesulfonate.

IV. TEST METHOD

A. Principle

The detection of forward mutations at the *ad-3* locus in either homokaryons or heterokaryons may be used. However, use of two component heterokaryons is recommended because of the greater range of mutations which can be recovered. In either case, the test relies on the identification of purple (mutant) colonies among a large number of white (wild-type) colonies. A representative sample of purple colonies can be recovered and thoroughly analyzed genetically.

B. Description

Forward mutations at the *ad-3* locus can be detected using noncolonial strains of *N. crassa* grown on media containing sorbose as well as glucose. Under these conditions, colonies are formed and reproducible colonial morphology results. Adenine-requiring mutants which accumulate a reddish-purple pigment can be readily identified and counted.

C. Strain selection

1. Designation

At the present time, heterokaryon 12 is recommended for use in this assay. The use of other strains may also be appropriate.

2. Preparation and storage

Stock culture preparation and storage, growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented.

3. Media

Frie's No. 3 minimal medium or Westgaard's Synthetic medium with 1.5% agar or any medium known to support growth and characteristic colonial morphology may be used in the assay.

D. Preparation of conidia

Stock cultures should be grown on minimal medium to select for single colonies with noncolonial morphology. Single colony isolates then should be inoculated into agar flasks and incubated at 35 C for 48 hrs to select colonies with spreading growth patterns in which mycelia cover the entire flask. Flasks should be incubated at 23-25 C and those with bright orange conidia selected for preparation of conidial suspensions. Suspensions should be diluted for use in distilled water.

E. Metabolic activation

Conidia should be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

F. Control groups

Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation should be included in each experiment.

G. Test chemicals

1. Vehicle

Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

2. Exposure concentrations

a. The test should initially be performed over a broad range of concentrations selected on the basis of a preliminary assay. Effective treatment times should also be selected in the preliminary assay.

b. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. For toxic chemicals, the highest concentration tested should not reduce survival below 10% of that seen in the control cultures. Relatively insoluble chemicals should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

c. Each test should include five treatment points; two at fixed concentrations for different time periods, and three at varying concentrations for fixed periods of time.

d. When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations.

V. TEST PERFORMANCE

A. Treatment

1. Growing or nongrowing conidia should be exposed to the test chemical with and without metabolic activation. At the end of the exposure period, treatment should be terminated by chemical quenching. The quenching solution may contain 0.1% sodium thiosulfate.

2. Conidia should then be plated on the appropriate media to determine mutation induction and viability. At the end of the incubation period, colonies should be scored for viability and mutation induction.

3. Mutants should be classified according to color and morphology.

4. Both mutation frequency and viability should be determined both immediately before and immediately after chemical treatment.

B. Incubation conditions

All plates in a given test should be incubated for the same time period. This incubation period may be from 2-7 days at 30 C.

C. Number of cultures

Generally, fifteen to 20 individual plates per concentration should be used.

VI. DATA AND REPORT

A. Treatment of results

Individual plate counts for test substance and controls should be presented for both mutation induction and survival. The mean number of colonies per plate and standard deviation should be presented. Data should be presented in tabular form indicating, as applicable, numbers of colonies counted, numbers of mutants identified and classification of mutants (e.g, color segregants). Sufficient detail should be provided for verification of survival and mutation frequencies.

B. Statistical evaluation

Data should be evaluated by appropriate statistical techniques.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of mutant colonies. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of mutant colonies or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results from the *ad-3* system in *N. crassa* indicate that, under the test conditions, the test substance causes mutations in the DNA of this organism.

2. Negative results indicate that under the test conditions the test substance is not mutagenic in *N. crassa.*

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. strain of organism used in the assay;

2. test chemical vehicle, doses used and rationale for dose selection;

3. method used for preparation of conida;

4. treatment conditions, including length of exposure and method used to stop treatment;

5. incubation times and temperature;

6. details of both the protocol used to prepare the metabolic activation system and of its use in the assay;

7. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Brockman HE, de Serres FJ. 1963. Induction of ad-3 mutants of *Neurospora crassa* by 2-aminopurine. Genetics 48: 597-604.

2. de Serres FJ, Malling HV. 1971. Measurement of recessive lethal damage over the entire genome and at two specific loci in the ad-3 region of a two-component heterokaryon of *Neurospora crassa*. In: Chemical mutagens: principles and methods for their detection, Vol. 2. Hollaender A, ed. New York and London: Plenum Press, pp. 311-342.

3. Matzinger PK, Ong T-M. 1976. In vitro activation of aflatoxin B_1 to metabolites mutagenic in *Neurospora crassa*. Mutation Research 37:27-32.

HG-Gene Muta-Insects
October, 1983

SEX-LINKED RECESSIVE LETHAL TEST IN *DROSOPHILA MELANOGASTER*

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I. PURPOSE

The sex-linked recessive lethal (SLRL) test using *Drosophila melanogaster* detects the occurrence of mutations, both point mutations and small deletions, in the germ line of the insect. This test is a forward mutation assay capable of screening for mutations at about 800 loci on the X-chromosome. This represents about 80% of all X-chromosome loci. The X-chromosome represents approximately one-fifth of the entire haploid genome.

II. DEFINITIONS

A. Lethal mutation is a change in the genome which, when expressed, causes death to the carrier.

B. Recessive mutation is a change in the genome which is expressed in the homozygous or hemizygous condition.

C. Sex-Linked genes are present on the sex (X or Y) chromosomes. Sex-linked genes in the context of this guideline refer only to those located on the X-chromosome.

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, ethyl methanesulfonate or N-nitroso-dimethylamine.

IV. TEST METHOD

A. Principle

Mutations in the X-chromosome of *D. melanogaster* are phenotypically expressed in males carrying the mutant gene. When the mutation is lethal in the hemizygous condition, its presence is inferred from the absence of one class of male offspring out of the two that are normally produced by a heterozygous female. The SLRL test takes advantage of these facts by means of specially marked and arranged chromosomes.

B. Description

Wild-type males are treated and mated to appropriate females. Female offspring are mated individually to their brothers, and in the next generation the progeny from each separate dose are scored for phenotypically wild-type males. Absence of these males indicates that a sex-linked recessive lethal mutation has occurred in a germ cell of the P_1 male.

C. Drosophila stocks

Males of a well-defined wild type stock and females of the Muller-5 stock may be used. Other appropriately marked female stocks with multiply inverted X-chromosomes may also be used.

D. Control groups

1. Concurrent controls

Concurrent positive and negative (vehicle) controls should be included in each experiment.

2. Positive controls

Examples of positive controls include ethyl methanesulfonate and N-nitroso-dimethylamine.

3. Other positive controls

Other positive control reference substances may be used.

4. Negative controls

Negative (vehicle) controls should be included. The size of the negative (vehicle) control group should be determined by the availability of appropriate labortory historical control data. If the historical control data are of sufficient numbers, concurrent controls may not be necessary.

E. Test chemicals

1. Vehicle

Test chemicals should be dissolved in water. Compounds which are insoluble in water may be dissolved or suspended in appropriate vehicles (e.g., a mixture of ethanol and Tween-60 or 80) and then diluted in water or saline prior to administration. Dimethylsulfoxide should be avoided as a vehicle.

2. Dose levels

For the initial assessment of mutagenicity, it may be sufficient to test a single dose of the test substance. This dose should be the maximum tolerated dose or that which produces some indication of toxicity. If the test is being used to verify mutagenic activity in this system at least two additional exposure levels should be used.

3. Route of administration

Exposure may be oral, by injection or by exposure to gases or vapors. Feeding of the test compound may be done in sugar solution. When necessary, substances may be dissolved in 0.7% NaCl solution and injected into the thorax or abdomen.

V. TEST PERFORMANCE

A. Treatment and mating

Wild-type males (3-5 days old) should be treated with the test substance and mated individually to an excess of virgin females from the Muller-5 stock or females from another appropriately marked (with multiply-inverted X-chromosomes) stock. The females should be replaced with fresh virgins every 2-3 days to cover the entire germ cell cycle. The offspring of these females are scored for lethal effects corresponding to the effects on mature sperm, mid or late stage spermatids, early spermatids, spermatocytes and spermatogonia at the time of treatment.

B. Fl matings

Heterozygous F_1 females from the above crosses should be allowed to mate individually (i.e. one female per vial) with their brothers. In the F_2 generation, each culture should be scored for the absence of wild-type males. If a culture appears to have arisen from an F_1 female carrying a lethal in the parental X-chromsome (i.e. no males with the treated chromosome are observed), daughters of that female with the same genotype should be tested to ascertain if the lethality is repeated in the next generation.

C. Number of matings

1. The test should be designed with a predetermined sensitivity and power. The number of flies in each group should reflect these defined parameters. The spontaneous mutant frequency observed in the appropriate control group will strongly influence the number of treated chromosomes that must be analysed to detect substances which show mutation rates close to those of the controls.

2. Test results should be confirmed in a separate experiment.

VI. DATA AND REPORT

A. Treatment of results

Data should be tabulated to show the number of chromosomes tested, the number of nonfertile males and the number of lethal chromosomes at each exposure concentration and for each mating period for each male treated. Numbers of clusters of different size per male should be reported.

B. Statistical evaluation

Data should be evaluated by appropriate statistical techniques.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of sex-lined recessive lethals. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce neither a statistically significant dose-related increase in the number of sex-linked recessive lethals nor a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results in the SLRL test in *D. melanogaster* indicate that under the test conditions the test agent causes mutations in germ cells of this insect.

2. Negative results indicate that under the test conditions the test substance is not mutagenic in *D. melanogaster*.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subparts, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported.

1. *Drosophila* stock used in the assay, age of insects, number of males treated, number of sterile males, number of F_2 cultures established, number of F_2 cultures without progeny;

2. test chemical vehicle, treatment and sampling schedule, exposure levels, toxicity data, negative (vehicle) and positive controls, if appropriate;

3. criteria for scoring lethals;

4. number of chromosomes tested, number of chromosomes scored, numer of chromosomes carrying a lethal mutation;

5. historical control data, if available;

6. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Sobels FH, Vogel E. 1976. The capacity of Drosophila for detecting relevant genetic damage. Mutation Research 41:95-106.

2. Wurgler FE, Sobels FH, Vogel E. 1977. Drosophila as assay system for detecting genetic changes. In: Handbook of mutagenicity test procedures. Kilbey BJ, Legator M, Nichols W, Ramel C, eds. Amsterdam: Elsevier/North Holland Biomedical Press, pp. 335-373.

DETECTION OF GENE MUTATIONS IN SOMATIC CELLS IN CULTURE

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HG-Gene Muta-Somatic Cells
October, 1983

I. PURPOSE

Mammalian cell culture systems may be used to detect mutations induced by chemical substances. Widely used cell lines include L5178Y mouse lymphoma cells and the CHO and V-79 lines of Chinese hamster cells. In these cell lines the most commonly used systems measure mutation at the thymidine kinase (TK), hypoxanthine-guanine-phosphoribosyl transferase (HPRT) and Na^+/K^+ ATPase loci. The TK and HPRT mutational systems detect base pair mutations, frameshift mutations and small deletions; the Na^+/K^+ ATPase system detects base pair mutations only.

II. DEFINITIONS

A. A forward mutation assay detects a gene mutation from the parental type to the mutant form which gives rise to a change in an enzymatic or functional protein.

B. Base pair mutagens are agents which cause a base change in the DNA.

C. Frameshift mutagens are agents which cause the addition or deletion of single or multiple base pairs in the DNA molecule.

D. Phenotypic expression time is a period during which unaltered gene products are depleted from newly mutated cells.

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, ethyl methanesulfonate, N-nitroso-dimethylamine, 2-acetylaminofluorene, 7,12-dimethylbenzanthracene or hycanthone.

IV. TEST METHOD

A. Principle

Cells are exposed to test substance, both with and without metabolic activation, for a suitable period of time and subcultured to determine cytotoxicity and to allow phenotypic expression prior to mutant selection.

Cells deficient in thymidine kinase (TK) due to the forward mutation TK^+. TK^- are resistant to the cyotoxic effects of pyrimidine analogues such as bromodeoxyuridine (BrdU), fluorodeoxyuridine (FdU) or trifluorothymidine (TFT). The deficiency of the "salvage" enzyme thymidine kinase means that these antimetabolites are not incorporated into cellular nucleotides and the nucleotides needed for cellular metabolism are obtained solely from de novo synthesis. However, in the presence of thymidine kinase, BrdU, FdU or TFT are incorporated into the nucleotides, resulting in inhibition of cellular metabolism and cytotoxicity. Thus mutant cells are able to proliferate in the presence of BrdU, FdU or TFT whereas normal cells, which contain thymidine kinase, are not. Similarly cells deficient in HPRT are selected by resistance to 8-azaguanine (AG) or 6-thioguanine (TG) and cells with altered Na^+/K^+ ATPase are selected by resistance to ouabain.

B. Description

Cells in suspension or monolayer culture are exposed to the test substance, both with and without metabolic activation, for a defined period of time. Cytotoxicity is determined by measuring the colony forming ability or growth rate of the cultures after the treatment period. The treated cultures are maintained in growth medium for a sufficient period of time - characteristic of each selected locus - to allow near-optimal phenotypic expression of induced mutations. Mutant frequency is determined by seeding known numbers of cells in medium containing the selective agent to detect mutant cells, and in medium without selective agent to determine the cloning efficiency. After a suitable incubation time, cell colonies are counted. The number of mutant colonies in selective medium is adjusted by the number of colonies in nonselective medium to derive the mutant frequency.

C. Cells

1. Type of cells used in the assay

A variety of cell lines are available for use in this assay including subclones of L5178Y, CHO cells or V-79 cells. Cell types used in this assay should have a demonstrated sensitivity to chemical mutagens, a high cloning efficiency and a low spontaneous mutation frequency. Cells should be checked for Mycoplasma contamination and may be periodically checked for karyotype stability.

2. Cell growth and maintenance

Appropriate culture media and incubation conditions (culture vessels, CO_2 concentrations, temperature and humidity) should be used.

D. Metabolic activation

Cells should be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

E. Control groups

Positive and negative (untreated and/or vehicle) controls should be included in each experiment. When metabolic activation is used, the positive control substance should be known to require such activation.

F. Test chemicals

1. Vehicle

Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. The final concentration of the vehicle should not interfere with cell viability or growth rate.

2. Exposure concentrations

(a) The test should be designed to have a predetermined sensitivity and power. The number of cells, cultures, and concentrations of test substance used should reflect these defined parameters. The number of cells per culture is based on the expected background mutant frequency; a general guide is to use a number which is ten times the inverse of this frequency.

(b) Several concentrations (usually at least 4) of the test substance should be used. These should yield a concentration-related toxic effect. The highest concentration should produce a low level of survival and the survival in the lowest concentration should approximate the negative control. Cytotoxicity should be determined after treatment with the test substance both in the presence and in the absence of an exogenous metabolic activation system. Relatively insoluble substances should be tested up to their limit of solubility under culture conditions. For freely-soluble non-toxic substances the highest concentration used should be determined on a case-by-case basis.

V. TEST PERFORMANCE

A. Cells should be exposed to the test substance both with and without metabolic activation. Exposure should be for a suitable period of time, in most cases 1 to 5 hours is effective; exposure time may be extended over one or more cell cycles.

B. At the end of the exposure period, cells should be washed and cultured to determine viability and to allow for expression of the mutant phenotype.

C. At the end of the expression period, which should be sufficient to allow near optimal phenotypic expression of induced mutants, cells should be grown in medium with and without selective agent(s) for determination of numbers of mutants and cloning efficiency respectively.

D. Results should be confirmed in an independent experiment.

VI. DATA AND REPORT

A. Treatment of results

Data should be presented in tabular form. Individual colony counts for the treated and control groups should be presented for both mutation induction and survival.

Survival and cloning efficiencies should be given as a percentage of the controls. Mutant frequency should be expressed as number of mutants per number of surviving cells.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant concentration-related increase in the mutant frequency. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

2. A test substance which does not produce either a statistically significant concentration-related increase in the mutant frequency or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results for an in vitro mammalian cell gene mutation test indicate that, under the test conditions, a substance induces gene mutations in the cultured mammalian cells used.

2. Negative results indicate that, under the test conditions, the test substance does not induce gene mutations in the cultured mammalian cells used.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. cell type used, number of cell cultures, methods used for maintenance of cell cultures;
2. rationale for selection of concentrations and number of cultures;
3. test conditions: composition of media, CO_2 concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period (including number of cells seeded and subculture and feeding schedules, if appropriate), selective agent(s);
4. methods used to enumerate numbers of viable and mutant cells;
5. dose-response relationship, where possible.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Amacher DE, Paillet SC, Ray V. 1979. Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. I. Application to genetic toxicology testing. Mutation Research 64:391-406.

2. Amacher DE, Paillet SC, Turner GN, Ray VA, Salsburg VA. 1980. Point mutations at the thymidine kinase locus in L5178Y mouse lymphoma cells. II. Test validation and interpretation. Mutation Research 72:447-474.

3. Bradley MO, Bhuyan B, Francis MC, Langenback R, Peterson A, Huberman E. 1981. Mutagenesis by chemical agents in V-79 Chinese hamster cells: a review and analysis of the literature: a report of the Gene-Tox Program. Mutation Research 87:81-142.

4. Clive D, Johnson KO, Spector JFS, Batson AG, Brown MM. 1979. Validation and characterization of the L5178Y $TK^{+}/^{-}$ mouse lymphoma mutagen assay system. Mutation Research 59:61-108.

5. Clive D, Spector JFS. 1975. Laboratory procedures for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells. Mutation Research 31:17-29.

6. Hsie AW, Casciano DA, Couch DB, Krahn DF, O'Neill JP, Whitfield BL. 1981. The use of Chinese hamster ovary cells to quantify specific locus mutation and to determine mutagenicity of chemicals: a report of the U.S. EPA's Gene-Tox Program. Mutation Research 86:193-214.

HG-Gene Muta-Mammal
October, 1983

THE MOUSE VISIBLE SPECIFIC LOCUS TEST

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I. PURPOSE

The mouse visible specific locus test (MSLT) may be used to detect and quantitate mutations in the germ line of a mammalian species.

II. DEFINITIONS

A. A visible specific locus mutation is a genetic change that alters factors responsible for coat color and other visible characteristics of certain mouse strains.

B. The germ line is the cells in the gonads of higher eukaryotes which are the carriers of the genetic information for the species.

III. REFERENCE SUBSTANCES

Not applicable.

IV. TEST METHOD

A. Principle

1. The principle of the MSLT is to cross individuals who differ with respect to the genes present at certain specific loci, so that a genetic alteration involving the standard gene at any one of these loci will produce an offspring detectably different from the standard heterozygote. The genetic change may be detectable by various means, depending on the loci chosen to be marked.

2. Three variations of the method currently exist for detecting newly arising point mutations in mouse germ cells:

   a. the visible specific locus test using either 5 or 7 loci;
   b. the biochemial specific locus test using up to 20 enzymes; and
   c. the test for mutations at histocompatibility loci.

3. Of the three tests, the visible specific locus test has been most widely used in assessing genetic hazard due to environmental agents. It is the method described in this guideline.

B. Description

For technical reasons, males rather than females are generally treated with the test agent. Treated males are then mated to females which are genetically homozygous for certain specific visible marker loci. Offspring are examined in the next generation for evidence that a new mutation has arisen.

C. Animal selection

1. Species and strain

Mice are recommended as the test species. Male mice should be either $(C_3H \times 101)F_1$ or $(101 \times C_3H)F_1$ hybrids. Females should be T stock virgins.

2. Age

Healthy sexually mature animals should be used.

3. Number

A decision on the minimum number of treated animals should take into account the spontaneous variation of the biological characterization being evaluated. Other considerations should include:

a. the use either historical or concurrent controls;
b. the power of the test;
c. the minimal rate of induction required;
d. the use of positive controls; and
e. the level of significance desired.

4. Assignment to groups

Animals should be randomized and assigned to treatment and control groups.

D. Control groups

1. Concurrent controls

The use of positive or spontaneous controls are left to the discretion of the investigator. However, any laboratory which has had no prior experience with the test should, at its first attempt, produce a negative control sample of 20,000 and a positive control, using 100 mg/kg 1-ethyl-nitrosourea, in a sample of 5000 offspring.

2. Historical controls

Long term, accumulated spontaneous control data of 43/801,406 are available for comparative purposes.

E. Test chemicals

1. Vehicle

When possible, test chemicals should be dissolved or suspended in distilled water or isotonic saline buffered appropriately, if needed, for stability. Water insoluble chemicals should be dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test compound nor produce major toxic effects. Fresh preparations of the test chemical should be employed.

2. Dose levels

Usually, only one dose level need be tested. This should be the highest dose tolerated without toxic effects, provided that any temporary sterility induced due to elimination of spermatagonia is of only moderate duration, as determined by a return of males to fertility within 80 days after treatment.

3. Route of administration

Acceptable routes of administration include gavage, inhalation, admixture with food or water, and IP or IV injections.

V. TEST PERFORMANCE

A. Treatment and mating

Hybrid F_1 (C_3H X 101 or 101 X C_3H) male mice should be treated with the test substance and immediately mated to virgin T stock females. Each treated male should be mated to a fresh group of 2-4 virgin females each week for 7 weeks, after which he should be returned to the first group of females and rotated through the seven sets of females repeatedly. This mating schedule generally permits sampling of all postspermatagonial stages of germ cell development during the first 7 weeks and rapid accumulation of data for exposed spermatagonial stem cells thereafter.

B. Examination of offspring

Offspring may be examined at (or soon after) birth but must be examined at about 3 week of age at which time the numbers of mutant and nonmutant offspring in each litter should be recorded.

Nonmutant progeny should be discarded. Mutant progeny should be subjected to genetic tests for verification.

VI. DATA AND REPORT

A. Treatment of results

Data should be presented in tabular form and should permit independent analysis of cell stage specific effects, and dose dependent phenomena. The data should be recorded and analyzed in such a way that clusters of identical mutations are clearly identified. The individual mutants detected should be thoroughly described. In addition, positive and negative control data, if they are available, should be tabulated so that it is possible to differentiate between concurrent (when available) and long term, accumulated mutation frequencies.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of specific locus mutations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of specific locus mutations or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results in the MSLT indicate that under the test conditions the test substance induces heritable gene mutations in the test species.

2. Negative results indicate that under the test conditions the test substance does not induce heritable gene mutations in the test species.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. strain, age and weight of animals used, number of animals of each sex in experimental and control groups;

2. test chemical vehicle, doses used and rationale for dose selection, toxicity data;

3. route and duration of exposure;
4. mating schedule;
5. time of examination for mutant progeny;
6. criteria for scoring mutants;
7. use of concurrent or negative controls;
8. dose response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provides a background of information on which this section is based. They should not be considered the only sources of information on test performance, however.

1. Russell LB, Selby PB, von Halle E, Sheridan W, Valcovic L. 1981. The mouse specific locus test with agents other than radiations: interpretation of data and recommendations for future work: a report of the Gene-Tox Program. Mutation Research 86:329-354.

HG-Chromo-In Vitro
October, 1983

IN VITRO MAMMALIAN CYTOGENETICS

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HG-Chromo-In Vitro
October, 1983

I. PURPOSE

The in vitro cytogenetics test is a mutagenicity test system for the detection of chromosomal aberrations in cultured mammalian cells. Chromosomal aberrations may be either structural or numerical. However, because cytogenetic assays are usually designed to analyse cells at their first post-treatment mitosis and numerical aberrations require at least one cell division to be visualized, this type of aberration is generally not observed in a routine cytogenetics assay. Structural aberrations may be of two types, chromosome or chromatid.

II. DEFINITIONS

A. Chromosome-type aberrations are changes which result from damage expressed in both sister chromatids at the same time.

B. Chromatid-type aberrations are damage expressed as breakage of single chromatids or breakage and/or reunion between chromatids.

III. REFERENCE SUBSTANCES

Not applicable.

IV. TEST METHOD

A. Principle

In vitro cytogenetics assays may employ cultures of established cell lines, cell strains or primary cell cultures. Cell cultures are exposed to the test substance both with and without metabolic activation. Following exposure of cell cultures to test substances, they are treated with a spindle inhibitor (e.g. colchicine or Colcemid) to arrest cells in a metaphase-like stage of mitosis (c-metaphase). Cells are then harvested and chromosome preparations made. Preparations are stained and metaphase cells are analyzed for chromosomal aberrations.

B. Description

Cell cultures are exposed to test compounds and harvested at various intervals after treatment. Prior to harvesting, cells are treated with a spindle inhibitor (e.g. colchicine or Colcemid®) to accumulate cells in c-metaphase. Chromosome preparations from cells are made, stained and scored for chromosomal aberrations.

C. Cells

1. Type of cells used in the assay

There are a variety of cell lines or primary cell cultures, including human cells, which may be used in the assay. Established cell lines and strains should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

2. Cell growth and maintenance

Appropriate culture media, and incubation conditions (culture vessels CO_2 concentrations, temperature and humidity should be used.

D. Metabolic activation

1. Cells should be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

E. Control groups

Positive and negative (untreated and/or vehicle) controls both with and without metabolic activation should be included in each experiment. When metabolic activation is used, the positive control substance should be known to require such activation.

F. Test chemicals

1. Vehicle

Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. Final concentration of the vehicle should not interfere with cell viability or growth rate.

2. Exposure concentrations

Multiple concentrations of the test substance over a range adequate to define the response should be tested. The highest test substance concentration tested with and without metabolic activation should show evidence or cytotoxicity or reduced mitotic activity. Relatively insoluble substances should be tested up to the limit of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

V. TEST PERFORMANCE

A. Established cell lines and strains

Prior to use in the assay, cells should be generated from stock cultures, seeded in culture vessels at the appropriate density and incubated at 37 C.

B. Human lymphocyte cultures

Heparinized or acid-citrate-dextrose whole blood should be added to culture medium containing a mitogen, e.g. phytohemagglutinin (PHA) and incubated at 37 C. White cells sedimented by gravity (buffy coat) or lymphocytes which have been purified on a density gradient may also be utilized.

C. Treatment with test substance

For established cell lines and strains, cells in the exponential phase of growth should be treated with test substances in the presence and absence of a metabolic activation system. Mitogen-stimulated human lymphocyte cultures may be treated with the test substance in a similar manner.

D. Number of cultures

At least two independent cultures should be used for each experimental point.

E. Culture harvest time

1. For established cell lines and strains multiple harvest times are recommended. However, for screening purposes, a single harvest time, e.g. 24 hrs, may be appropriate. If the test chemical changes the cell cycle length, the fixation intervals should be changed accordingly.

2. For human lymphocyte cultures, the substance to be tested may be added to the cultures at various times after mitogen stimulation so that there is a single harvest time after the initiation of the cell culture. Alternatively, a single treatment may be followed by multiple harvest times. Harvest time should be extended for those chemicals which induce an apparent cell cycle delay. Because the population of human lymphocytes is only partially synchronized, a single treatment, at, or close to, the time when metaphase stages first appear in the culture will include cells in all phases of the division cycle. Therefore, a single harvest at the time of second mitosis may be carried out for screening purposes.

3. Cell cultures are treated with a spindle inhibitor, (e.g. colchicine or Colcemid) one or two hours prior to harvesting. Each culture is harvested and processed separately for the preparation of chromosomes.

F. Chromosome preparation

Chromosome preparation involves hypotonic treatment of the cells, fixation and staining.

G. Analysis

Slides should be coded before analysis. The number of cells to be analysed should be based upon the spontaneous control frequency, defined sensitivity and the power chosen for the test before analysis. In human lymphocytes, only cells containing 46 centromeres should be analysed. In established cell lines and strains only metaphases containing ± 2 centromeres of the modal number should be analysed. Uniform criteria for scoring aberrations should be used.

VI. DATA AND REPORT

A. Treatment of results

Data should be presented in a tabular form. Different types of structural chromosomal aberrations should be listed with their numbers and frequencies for experimental and control groups. Data should be evaluated by appropriate statistical methods. Gaps or achromatic lesions are recorded separately and not included in the total aberration frequency.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of structural chromosomal aberrations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of structural chromosomal aberrations or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results in the in vitro cytogenetics assay indicate that under the test conditions the test substance induces chromosomal aberrations in cultured mammalian somatic cells.

2. Negative results indicate that under the test conditions the test substance does not induce chromosomal aberrations in cultured mammalian somatic cells.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. cells used, density and passage number at time of treatment, number of cell cultures;

2. methods used for maintenance of cell cultures including medium, temperature and CO_2 concentration;

3. test chemical vehicle, concentration and rationale for the selection of the concentrations used in the assay, duration of treatment;

4. details of both the protocol used to prepare the metabolic activation system and of its use in the assay;

5. identity of spindle inhibitor, its concentration and duration of treatment;

6. time of cell harvest;

7. positive and negative controls;

8. methods used for preparation of slides for microscopic examination;

9. number of metaphases analysed;

10. mitotic index where applicable;

11. criteria for scoring aberrations;

12. type and number of aberrations, given separately for each treated and control culture, total number of aberrations per group; frequency distribution of number of chromosomes in established cell lines and strains;

13. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

2. Evans HJ. 1976. Cytological methods for detecting chemical mutagens. In: Chemical mutagens, principles and methods for their detection. Vol.4. Hollaender A, ed. New York, London: Plenum Press. pp. 1-29.

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4. Ishidate M Jr, Odashima S. 1975. Chromosome tests with 134 compounds on Chinese hamster cells in vitro: a screening for chemical carcinogens. Mutation Research 48:337-354.

5. Preston RJ, Au W, Bender MA, Brewen JG, Carrano AV, Heddle JA, McFee AF, Wolff S, Wassom JS. 1981. Mammalian in vivo and in vitro cytogenetic assays: a report of the Gene-Tox Program. Mutation Research 87:143-188.

HG-Chromo-Bone Marrow
October, 1983

IN VIVO MAMMALIAN BONE MARROW CYTOGENETICS TESTS: CHROMOSOMAL ANALYSIS

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

The *in vivo* bone marrow cytogenetic test is a mutagenicity test for the detection of structural chromosomal aberrations. Chromosomal aberrations are generally evaluated in first post-treatment mitoses. With the majority of chemical mutagens, induced aberrations are of the chromatid type but chromosome type aberrations also occur.

II. DEFINITIONS

A. Chromosome-type aberrations are changes which result from damage expressed in both sister chromatids at the same time.

B. Chromatid-type aberrations are damage expressed as breakage of single chromatids or breakage and/or reunion between chromatids.

III. REFERENCE SUBSTANCES

Not applicable.

IV. TEST METHOD

A. Principle

Animals are exposed to test chemicals by appropriate routes and are sacrificed at sequential intervals. Chromosome preparations are made from bone marrow cells. The stained preparations are examined and metaphase cells are scored for chromosomal aberrations.

B. Description

The method employs bone marrow of laboratory rodents which have been exposed to test chemicals. Prior to sacrifice, animals are further treated with a spindle inhibitor, (e.g. colchicine or Colcemid®) to arrest the cells in c-metaphase. Chromosome preparations from the cells are stained and scored for chromosomal aberrations.

C. Animal selection

1. Species and strain

Any appropriate mammalian species may be used. Examples of commonly used rodent species are rats, mice, and hamsters.

2. Age

Healthy young adult animals should be used.

3. Number and sex

At least five female and five male animals per experimental and control group should be used. Thus, ten animals would be sacrificed per time per group treated with the test compound if several test times after treatment are included in the experimental schedule. The use of a single sex or different number of animals should be justified.

4. Assignment to groups

Animals should be randomized and assigned to treatment and control groups.

D. Control groups

1. Concurrent controls

Concurrent positive and negative (vehicle) controls should be included in the assay.

2. Positive controls

A single dose positive control showing a significant response at any one time point is adequate. A compound known to produce chromosomal aberrations in vivo should be employed as the positive control.

E. Test chemicals

1. Vehicle

When possible, test chemicals should be dissolved in isotonic saline or distilled water. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test compound should be employed.

2. Dose levels

For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose or that producing some indication of cytotoxicity (e.g. partial inhibition of mitosis). Additional dose levels may be used. For determination of dose-response, at least three dose levels should be used.

3. Route of administration

The usual routes are oral or by intraperitoneal injection. Other routes may be appropriate.

4. Treatment schedule

In general, test substances should be administered once only. However, based on toxicological information a repeated treatment schedule may be employed.

V. TEST PERFROMANCE

A. Generally the test may be performed in two assay:

1. Animals are treated with the test substance once at the selected dose(s). Samples are taken at three times after treatment. For rodents, the central sampling interval is 24 hours. Since cell cycle kinetics can be influenced by the test substance, one earlier and one later sampling interval adequately spaced within the range of 6 to 48 hours should be applied. Where the additional dose levels are tested in a subsequent experiment, samples should be taken at the predetermined most sensitive interval or, if this is not established, at the central sampling time.

2. If a repeated treatment schedule is used at the selected dose(s), samples should be taken 6 and 24 hours after the last treatment; other sampling times may be used if justified. Where the additional dose levels are tested in a subsequent

experiment, samples should be taken at the predetermined most sensitive interval or, if this is not established, at six hours after the last treatment.

B. Administration of spindle inhibitor

Prior to sacrifice, animals should be injected IP with an appropriate dose of a spindle inhibitor (e.g. colchicine or Colcemid®) to arrest cells in c-metaphase.

C. Preparation of slides

Immediately after sacrifice, the bone marrow should be obtained, exposed to hypotonic solution, and fixed. The cells should then be spread on slides and stained. Chromosome preparations should be made following standard procedures.

D. Analysis

The number of cells to be analysed per animal should be based upon the number of animals used, the negative control frequency, the predetermined sensitivity and the power chosen for the test. Slides should be coded before microscopic analysis.

VI. DATA AND REPORT

A. Treatment of results

Data should be presented in tabular form for both cells and animals. Different types of structural chromosomal aberrations should be listed with their numbers and a mean frequency per cell for each animal in all treated and control groups. Gaps (achromatic lesions) should be recorded separately and not included in the total aberration frequency. Differences among animals within each group should be considered before making comparisons between treated and control groups.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of structural chromosomal aberrations or abnormal metaphase figures. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of chromosomal aberrations or abnormal metaphase figures or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results in the in vivo bone marrow cytogenetics assay indicate that under the test conditions the test substance induces chromosomal aberrations in the bone marrow of the test species.

2. Negative results indicate that under the test conditions, the test substance does not induce chromosomal aberrations in the bone marrow of the test species.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. species, strain, age, weight, number and sex of animals in each treatment and control group;

2. test chemical vehicle, dose levels used, rationale for dose selection;

3. route of administration, treatment and sampling schedules, toxicity data, negative and positive controls;

4. identity of spindle-inhibitor, its concentration and duration of treatment.

5. details of the protocol used for chromosome preparation, number of cells scored per animal, type and number of aberrations given separately for each treated and control animal;

6. mitotic index, where applicable;

7. criteria for scoring aberrations;

8. number and frequency of aberrant cells per animal in each treatment and control groups;

9. total number of aberrations per group;

10. number of cells with aberrations per group;

11. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Adler ID, Ramarao G, Epstein SS. 1971. In vivo cytogenetic effects of trimethyl-phosphate and of TEPA on bone marrow cells of male rats. Mutation Research 13:263-273.

2. Evans HJ. 1976. Cytological methods for detecting chemical mutagens. In: Chemical mutagens: principles and methods for their detection, Vol. 4. Hollaender A, ed. New York and London: Plenum Press, pp. 1-29.

3. Kilian JD, Moreland FE, Benge MC, Legator MS, Whorton EB Jr. 1977. A collaborative study to measure intralaboratory variation with the in vivo bone marrow metaphase procedure. In: Handbook of mutagenicity test procedures. Kilby BJ, Legator M, Nichols C, Ramel C, eds. Amsterdam: Elsevier/North_Holland Biomedical Press, pp. 243-260.

4. Preston JR, Au W, Bender MA, Brewen JG, Carrano AV, Heddle JA, McFee AF, Wolff S, Wassom J. 1981. Mammalian in vivo and in vitro cytogenetics assays: report of the Gene-Tox Program. Mutation Research 87:143-188.

HG-Chromo-Sister Chrom-In Vitro
October, 1983

IN VITRO SISTER CHROMATID EXCHANGE ASSAY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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I. PURPOSE

The sister chromatid exchange (SCE) assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. The test may be performed in vitro, using, for example, rodent or human cells, or in vivo using mammals, for example, rodents such as mice, rats and hamsters.

II. DEFINITION

Sister chromatid exchanges represent reciprocal interchanges of the two chromatid arms within a single chromosome. These exchanges are visualized during the metaphase portion of the cell cycle and presumably require enzymatic incision, translocation and ligation of at least two DNA helices.

III. REFERENCE SUBSTANCES

Not applicable.

IV. TEST METHOD

A. Principle

Following exposure of cell cultures to test chemicals, they are allowed to replicate in the presence of bromodeoxyuridine (BrdU), followed by treatment with colchicine or colcemid to arrest cells in a metaphase-like stage of mitosis (c-metaphase). Cells are then harvested and chromosome preparations made. Preparations are stained and metaphase cells analyzed for SCEs.

B. Description

In vitro SCE assays may employ monolayer or suspension cultures of established cell lines, cell strains or primary cell cultures. Cell cultures are exposed to test chemical and are allowed to replicate in the presence of BrdU. Prior to harvest, cells are treated with a spindle inhibitor (e.g. Colchicine or Colcemid ®) to accumulate cells in c-metaphase. Chromosome preparations from cells are made, stained and analyzed for SCEs.

C. Cells

1. Type of cells used in the assay

There are a variety of cell lines or primary cell cultures, including human cells, which may be used in the assay. Established cell lines and strains should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

2. Cell growth and maintenance

Appropriate culture media and incubation conditions (culture vessels, temperature, and humidity CO_2 should be used.

D. Metabolic activation

1. Cells should be exposed to test chemical both in the presence and absence of an appropriate metabolic activation system.

E. Control groups

1. Concurrent controls

Positive and negative (untreated and/or vehicle) controls, with and without metabolic activation, should be included in each experiment.

F. Test chemicals

1. Vehicle

Test substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. Final concentration of the vehicle should not cell viability or growth rate.

2. Exposure concentrations

Multiple concentrations of the test substance over a range adequate to define the response should be tested. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test substance may be altered in the presence of metabolic activation systems. Cytotoxicity may be evidenced by a large (e.g. 75%) decrease in the number of cells that have divided twice in the presence of BrdU. Relatively insoluble substances should be tested up to the limit of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis. When appropriate, a positive response should be confirmed by using a narrow range of test concentrations.

V. TEST PERFORMANCE

A. Established cell lines and strains

1. Prior to use in the assay, cells should be generated from stock cultures, seeded in culture vessels at the appropriate density and incubated at 37 C.

2. Cell lines and strains should be treated with test chemical both with and without metabolic activation when they are in the exponential stage of growth. At the end of the exposure period, cells should be washed and incubated for two replication cycles in medium containing BrdU. After BrdU is added, the cultures should be handled in darkness, under "safe" (e.g. darkroom) lights, or in dim light from incandescent lamps to minimize photolysis of BrdU containing DNA. At the end of the BrdU incubation period, cells should be fixed and stained for SCE determination. Cultures should be treated with a spindle inhibitor (e.g. colchicine or Colcemid®) 2 hr prior to harvesting.

B. Human lymphocyte cultures

1. For preparation of human lymphocyte cell cultures, heparinized or acid-citrate-dextrose treated whole blood should be added to culture medium containing a mitogen, e.g., phytohemagglutinin (PHA) and incubated at 37 C. White cells sedimented by gravity (buffy coat) or lymphocytes which have been purified on a density gradient such as Ficoll-Hypaque may also be utilized.

2. Cells should be exposed to the test chemical during at least two time intervals, e.g. G_0 and S. Exposure during the G_0 phase of the cell cycle should be accomplished by adding the test substance prior to addition of mitogen. Exposure during or after the first S phase may be accomplished by exposing cells 24-30 hrs after mitss, under "safe" (e.g. darkroom) lights, or in dim light from incandescent lamps to minimize photolysis of BrdU containing DNA. At the end of the BrdU incubation period, cells should be fixed and stained for SCE determination. Cultures should be treated with a spindle inhibitor (e.g. colchicine or Colcemid®) 2 hr prior to harvesting.

B. Human lymphocyte cultures

1. For preparation of human lymphocyte cell cultures, heparinized or acid-citrate-dextrose treated whole blood should be added to culture medium containing a mitogen, e.g., phytohemagglutinin (PHA) and incubated at 37 C. White cells sedimented by gravity (buffy coat) or lymphocytes which have been purified on a density gradient such as Ficoll-Hypaque may also be utilized.

2. Cells should be exposed to the test chemical during at least two time intervals, e.g. G_0 and S. Exposure during the G_0 phase of the cell cycle should be accomplished by adding the test substance prior to addition of mitogen. Exposure during or after the first S phase may be accomplished by exposing cells 24-30 hrs after mitogen stimulation. After exposure, cells should be washed and then cultured in the absence of the chemical.

C. Culture harvest time

A single harvest time, one that yields an optimal percentage of second division metaphases, is recommended. If there is reason to suspect that this is not a representative sampling time (which may occur for short-lived, cycle specific chemicals), then additional harvest times should be selected.

D. Staining method

Staining of slides to reveal SCEs can performed according to any of several protocols. However, the fluorescence plus Giemsa method is recommended.

E. Number of cultures

At least two independent cultures should be used for each experimental point.

F. Analysis

Slides should be coded before analysis. The number of cells to be analysed should be based upon the spontaneous control frequency and defined sensitivity and the power of the test chosen before analysis. In human lymphocytes, only cells containing 46 centromeres should be analysed. In established cell lines and strains, only metaphases containing ± 2 centromeres of the modal number should be analysed. Uniform criteria for scoring SCEs should be used.

VI. DATA AND REPORT

A. Treatment of results

Data should be presented in tabular form, providing scores for both the number of SCEs for each metaphase and the number of SCEs per chromosome for each metaphase.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determing a positive result, one of which is a statistically significant dose-related increase in the number of sister chromatid exchanges. Another criterion may be based upon detection of a reproducible and statistically signficiant positive response for at least one of the test substance concentrations.

2. A test substance which produces neither a statistically significant dose-related increase in the number of sister chormatid exchanges nor a statistically significant and reproducible positive response at any one of the test points is considered not to induce rearrangements of segments of DNA in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results in the in vitro SCE assay indicate that under the test conditions the test substance induces reciprocal chromatid interchanges in cultured mammalian somatic cells.

2. Negative results indicate that under the test conditions the test substance does not induce receiprocal chromatid interchanges in cultured mammalian somatic cells.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. cells used, density at time of treatment, number of cell cultures;

2. methods used for maintenance of cell cultures including medium, temperature and CO_2 concentration;

3. test chemical vehicle, concentration and rationale for the selection of the concentrations of test chemical used in the assay, duration of treatment;

4. details of both the protocol used preparation of the metabolic activation system and its use in the assay.

5. growth period in BrdU; identity of spindle inhibitor, its concentration and duration of treatment;

6. time of cell harvest;
7. positive and negative controls;
8. method used to prepare slides for SCE determination;
9. criteria for scoring SCEs;
10. details of the protocol used for growth and treatment of human cells if used in the assay;
11. dose-response relationship, if applicable.

VII. REFERENCES

The following reference may be helpful in developing acceptable protocols, and provides a background of information on which this section is based. It should not be considered the only source of information on test performance, however.

1. Latt SA, Allen J, Bloom SE, Carrano A, Falke E, Kram D, Schneider E, Schreck R, Tice R, Whitfield B, Wolff S. 1981. Sister chromatid exchanges: a report of the U.S. EPA's Gene-Tox Program. Mutation Research 87:17-62.

HG-Chromo-Sister Chrom-In Vivo
October, 1983

IN VIVO SISTER CHROMATID EXCHANGE ASSAY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

The sister chromatid exchange (SCE) assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. The test may be performed in vitro using cultured mammalian cells or in vivo using nonmammalian or mammalian tissues. The most commonly used assays employ bone marrow or lymphocytes from mammalian species such as mice, rats or hamsters. Human lymphocytes may also be used.

II. DEFINITION

Sister chromatid exchanges represent reciprocal interchanges of the two chromatid arms within a single chromosome. These exchanges are visualized during the metaphase portion of the cell cycle and presumably require enzymatic incision, translocation and ligation of at least two DNA helices.

III. REFERENCE SUBSTANCES

Not applicable.

IV. TEST METHOD

A. Principle

Animals are exposed to test substance by appropriate routes followed by administration of bromodeoxyuridine (BrdU). A spindle inhibitor (e.g. colchicine or Colecimid®) is administered prior to sacrifice. After sacrifice, tissue is obtained and metaphase preparations made, stained and scored for SCE.

B. Description

The method described here employs bone marrow of laboratory rodents exposed to test chemicals. After treatment with test chemical, animals are further treated with BrdU and, prior to sacrifice, with a spindle inhibitor (e.g. colchicine or Colcemid®) to arrest cells in c-metaphase. After sacrifice, chromosome preparations from bone marrow cells are made, stained and scored for SCE.

C. Animal selection

1. Species and strain

Any appropriate mammalian species may be used. Examples of commonly used rodent species include mice, rats, and hamsters.

2. Age

Healthy, young adult animals should be used.

3. Number and sex

At least five female and five male animals per experimental and control group should be used. The use of a single sex or different number of animals should be justified.

4. Assignment to groups

Animals should be randomized and assigned to treatment and control groups.

D. Control groups

1. Concurrent controls

Current positive and negative (vehicle) controls should be included in the assay.

2. Positive controls

A compound known to produce SCE in vivo should be employed as the positive control.

E. Test chemicals

1. Vehicle

When possible, test chemicals should be dissolved in isotonic saline or distilled water. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test compound nor produce toxic effects. Fresh preparations of the test compound should be employed.

2. Dose levels

For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose or that producing some indication of toxicity as evidenced by animal morbidity (including death) or target cell toxicity. The LD_{50} is a suitable guide. Additional dose levels may be used. For determination of dose-response, at least three dose levels should be used.

3. Route of administration

The usual routes of administration are IP or oral. Other routes may be appropriate.

4. Treatment schedule

In general, test substances should be administered only once. However, based upon toxicological information a repeated treatment schedule may be employed.

V. TEST PERFORMANCE

A. Treatment

Animals should be treated with test chemical followed by administration of BrdU. BrdU may be administered by multiple IP injections, by continuous tail vein infusion or by subcutaneous implantation of tablets. Animals should be treated with a spindle inhibitor (e.g. colchicine or Colcemid®) 2 hr prior to sacrifice. After sacrifice, bone marrow should be extracted and slides made and prepared for SCE evaluation.

B. Staining method

Staining of slides to reveal SCEs can be performed according to any of several protocols. However, the fluorescence plus Giemsa method is recommended.

C. Number of cells scored

The number of cells to be analysed per animal should be based upon the number of animals used, the negative control frequency, the predetermined sensitivity and the power chosen for the test. Slides should be coded before microscopic analysis.

VI. DATA AND REPORT

A. Treatment of results

Data should be presented in tabular form, providing scores for both the number of SCE for each metaphase and the number of SCE per chromosome for each metaphase. Differences among animals within each group should be considered before making comparisons between treated and control groups.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretion of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of SCE. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of SCE or a statistically significant and reproducible positive response at any one of the test points is considered not to induce rearrangements of DNA segments in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results in the in vivo SCE assay indicate that under the test conditions the test substance induces reciprocal interchanges in the bone marrow of the test species.

2. Negative results indicate that under the test conditions the test substance does not induce reciprocal interchanges in the bone marrow of the test species.

E. Test Report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. species, strain, age, weight, number and sex of animals in each treatment and control group;
2. test chemical vehicle, dose level used, rationale for dose selection, toxicity data, negative and positive controls;
3. route and schedule of administration of both test chemical and BrdU;
4. identity of spindle inhibitor, its concentration and duration of treatment;
5. time of sacrifice after administration of BrdU;
6. details of the protocol used for slide preparation;
7. criteria for scoring SCE;
8. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Allen JW, Shuler CF, Latt SA. 1978. Bromodeoxyuridine tablet methodology for in vivo studies of DNA synthesis. Somatic Cell Genetics 4:393-405.

2. Allen JW, Shuler CF, Mendes RW, Latt SA. 1977. Simplified technique for in vivo analysis of sister chromatid exchanges using 5-bromodeoxyuridine tablets. Cytogenetics Cell Genetics 18:231-237.

3. Latt SA, Allen J, Bloom SE, Carrano A, Falke E, Kram D, Schneider E, Schreck R, Tice R, Whitfield B, Wolff S. 1981. Sister chromatid exchanges: a report of the Gene-Tox Program. Mutation Research 87:17-62.

HG-Chromo-Micronuc
October, 1983

IN VIVO MAMMALIAN BONE MARROW CYTOGENETICS TESTS: MICRONUCLEUS ASSAY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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I. PURPOSE

The micronucleus test is a mammalian in vivo test which detects damage of the chromosomes or mitotic apparatus by chemicals. Polychromatic erythrocytes in the bone marrow of rodents are used in this assay. When the erythroblast develops into an erythrocyte the main nucleus is extruded and may leave a micronucleus in the cytoplasm. The visualization of micronuclei is facilitated in these cells because they lack a nucleus. Micronuclei form under normal conditions. The assay is based on an increase in the frequency of micronucleated polychromatic erythrocytes in bone marrow of treated animals.

II. DEFINITION

Micronuclei are small particles consisting of acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm.

III. REFERENCE SUBSTANCES

Not applicable.

IV. TEST METHOD

A. Principle

Animals are exposed to test substance by an appropriate route. They are sacrificed, the bone marrow extracted and smear preparations made and stained. Polychromatic erythrocytes are scored for micronuclei under the microscope.

B. Description

The method employs bone marrow of laboratory mammals which are exposed to test substances.

C. Animal selection

1. Species and strain

Mice are recommended. However, any appropriate mammalian species may be used.

2. Age

Healthy young adult animals should be used.

3. Number and sex

At least five female and five male animals per experimental and control group should be used. Thus, 10 animals would be sacrificed per time per group if several test times after treatment were included in the experimental schedule. The use of a single sex or a different number of animals should be justified.

4. Assignment to groups

Animals should be randomized and assigned to treatment and control groups.

D. Control groups

1. Concurrent controls

Concurrent positive and negative (vehicle) controls should be included in each assay.

2. Positive controls

A compound known to produce micronuclei in vivo should be employed as the positive control.

E. Test chemicals

1. Vehicle

Solid and liquid test substances should be dissolved or suspended in distilled water or isotonic saline. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test compound nor produce toxic effects. Fresh preparations of the test compound should be employed.

2. Dose levels

For an initial assessment, one dose of the test substance may be used, the dose being the maximum tolerated dose or that producing some indication of cytotoxicity, e.g. a change in the ratio of polychomatic to normochromatic erythrocytes.

Additional dose levels may be used. For determination of dose response, at least three dose levels should be used.

3. Route of administration

The usual routes of administration are IP or oral. Other routes may be appropriate.

4. Treatment schedule

Test substances should generally be administered only once. However, based upon toxicological information a repeated treatment schedule may be employed.

V. TEST PERFORMANCE

A. Treatment and sampling times

1. Animals should be treated with the test substance once at the highest tolerated dose. Sampling times should coincide with the maximum response of the assay which varies with the test substance. Therefore, using the highest dose, bone marrow samples should be taken at least three times, starting not earlier than 12 hours after treatment, with appropriate intervals following the first sample but not extending beyond 72 hours. When other doses are used sampling should be at the maximum sensitive period, or, if that is not known, approximately 24 hours after treatment. Other appropriate sampling times may be used in addition.

2. If a repeated treatment schedule is used, samples should be taken at least three times, starting not earlier than 12 hours after the last treatment and at appropriate intervals following the first sample, but not extending beyond 72 hours.

3. Bone marrow should be obtained immediately after sacrifice. Cells should be prepared, put on slides, spread as a smear and stained.

B. ANALYSIS

Slides should be coded before microscopic analysis. At least 1000 polychromatic erthyrocytes per animal should be scored for the incidence of micronuclei. The ratio of polychromatic to normochromatic erythrocytes should be determined for each animal by counting a total of 1000 erythrocytes. Additional information may be obtained by scoring normochromatic erythrocytes for micronuclei.

VI. DATA AND REPORT

A. Treatment of results

Criteria for scoring micronuclei should be given. Individual data should be presented in a tabular form including positive and negative (vehicle) controls and experimental groups. The number of polychromatic erythrocytes scored, the number of micronucleated polychromatic erythrocytes, the percentage of micronucleated cells, the number of micronucleated normochromatic erythrocytes, and, if applicable, the percentage of micronucleated erythrocytes and the ratio of normochromatic to polychromatic erythrocytes should be listed separately for each experimental and control animal. Absolute numbers should be included if percentages are reported.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive response, one of which is a statistically significant dose related increase in the number of micronucleated polychromatic erythrocytes. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of micronucleated polychromatic erythrocytes or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. The results of the micronucleus test provide information on the ability of a chemical to induce micronuclei in polychromatic erythrocytes of the test species under the conditions of the test. This damage may have been the result of chromosomal damage or damage to the mitotic apparatus.

2. Negative results indicate that under the test conditions the test substance does not produce micronuclei in the bone marrow of the test species.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. species, strain, age, weight, number and sex of animals in each treatment and control group;

2. test chemical vehicle, dose levels used, rationale for dose selection;

3. rationale for and description of treatment and sampling schedules, toxicity data, negative and positive controls;

4. details of the protocol used for slide preparation;

5. criteria for identifying micronucleated erythrocytes;

6. dose-response relationship if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Cihak R. 1979. Evaluation of benzidine by the micronucleus test. Mutation Research 67:383-384.

2. Cole RJ, Taylor N, Cole J, Arlett CF. Short-term tests for transplacentally active carcinogens. 1. Micronucleus formation in fetal and maternal mouse erythroblasts. 1981. Mutation Research 80:141-157.

3. Kliesch U, Danford N, Adler ID. 1981. Micronucleus test and bone-marrow chromosome analysis. A comparison of 2 methods in vivo for evaluating chemically induced chromosomal alterations. Mutation Research 80:321-332.

4. Matter B, Schmid W. 1971. Trenimon-induced chromosomal damage in bone-marrow cells of six mammalian species, evaluated by the micronucleus test. Mutation Research 12:417-425.

5. Schmid W. 1975. The micronucleus test. Mutation Research 31:9-15.

6. Schmid W. 1976. The micronucleus test for cytogenetic analysis. In: Chemical mutagens, principles and methods for their detection, Vol. 4. Hollaender A, ed. New York and London: Plenum Press, pp. 31-53.

HG-Chromo-Insects
October, 1983

HERITABLE TRANSLOCATION TEST IN *DROSOPHILA MELANOGASTER*

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
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I. PURPOSE

The heritable translocation test in *Drosophila* measures the induction of chromosomal translocations in germ cells of insects. Stocks carrying genetic markers on two or more chromosomes are used to follow the assortment of chromosomes in meiosis. The F_1 male progeny of treated parents are individually mated to females and the F_2 progeny phenotypes are scored. The observed spectrum of phenotypes is used to determine the presence or absence of a translocation. This is usually indicated by a lack of independent assortment of genes on different chromosomes.

II. DEFINITIONS

A. Chromosome mutations are chromosomal changes resulting from breakage and reunion of chromosomes. Chromosomal mutations are also produced through nondisjunction of chromosomes during cell division.

B. Reciprocal translocations are chromosomal translocations resulting from reciprocal exchanges between two or more chromosomes.

C. Heritable translocations are reciprocal translocations transmitted from parent to the succeeding progeny.

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, ethyl methanesulfonate or N-dimethyl-nitrosamine.

IV. TEST METHOD

A. Principle

The method is based on the principle that balanced reciprocal chromosomal translocations can be induced by chemicals in the germ cells of treated flies and that these translocations are detected in the F_2 progeny using genetic markers (mutations). Different mutations may be used as genetic markers and two or more of the four chromosomes may be genetically marked for inclusion in this test.

B. Description

Wild-type males are treated with chemical and bred with females of known genetic markers. The F_1 males are collected and individually bred with virgin females of the female parental stock. The resulting F_2 progeny are scored. Putative translocation carriers are confirmed with an F_3 cross.

1. Illustrative example

The following example serves to illusrate the method. Males carrying genes for red eye color on chromosomes II and III are bred with females of white eye color carrying alleles for brown (bw) on the second chromosome and scarlet (st) and pink (pp) on the third chromosome. The F_1 male progeny are bred with virgin females of the female parental stock and the resulting F_2 progeny are examined for eye color phenotypes. If there is no translocation in the F_1 male, then the resulting F_2 progeny will have four eye color phenotypes: red, white, orange, and brown. If the F_1 male carries a translocation between chromosomes II and III, only red and white-eye phenotypes are obtained in the F_2 generation. This happens because the F_1 translocation heterozygote produces two balanced (carrying either the parental or the translocated configuration of markers) and two unbalanced gametes. The unbalanced gametes (carrying one normal and one translocated chromosome) are unable to develop into normal individuals in the F_2 generation.

C. Drosophila stocks

Wild-type males and females of the genotype bw:st:pp (white eyes) may be used in the heritable translocation test. Other appropriately marked Drosophila stocks may also be used.

D. Control groups

Concurrent positive and negative (vehicle) controls should be included in each experiment.

Negative (vehicle) controls should be included. The size of the negative (vehicle) control group should be determined by the availability of appropriate laboratory historical control data.

If the historical control data are of sufficient numbers, concurrent controls may not be necessary.

E. Test chemicals

1. Vehicle

Test chemicals should be dissolved in water. Compounds which are insoluble in water may be dissolved or suspended in appropriate vehicles (e.g., a mixture of ethanol and Tween-60 or 80), and then diluted in water or saline prior to administration. Dimethylsulfoxide should be avoided as a vehicle.

2. Dose levels

For the initial assessment of mutagenicity, it may be sufficient to test a single dose of the test substance. This dose should be the maximum tolerated dose or that which produces some indication of toxicity. If the test is being used to verify mutagenic activity, at least two additional exposure levels should be used.

3. Route of administration

Exposure may be oral, by injection or by exposure to gases or vapours. Feeding of the test compound may be done in sugar solution. When necessary, substances may be dissolved in 0.7% NaCl solution and injected into the thorax or abdomen.

V. TEST PERFORMANCE

A. P1 mating

1. In the primary screen of a chemical, it is enough to sample one germ cell stage, either mature sperm or spermatids (for indirect acting mutagens). Other stages may be sampled if needed, i.e. when mature germ cells give a positive result and data from earlier germ cells are needed for the purpose of risk assessment. Thus, the treated males may be mated only once for a period of 3 days to sample sperm or transferred every 2-3 days to cover the entire germ cell cycle.

2. Mass matings may be performed because the control rate for translocations in the available literature is very low (near 0) and clustered events are extremely rare. Mated females may be aged for 2 weeks in order to recover an enhanced incidence of translocation due to the storage effect. The females are then allowed to lay eggs and F_1 males are collected for test mating.

B. F1 mating

F_1 males should be bred with virgin females of the parental female stock. Since each F_1 male represents one treated gamete of the male parent, the F_1 males have to be mated individually to virgin females. Each F_1 male should be mated to three females to ensure sufficient progeny.

C. Scoring the F2 generation

F_2 cultures (each representing 1 F_1 male tested) should be scored for the presence or absence of phenotype variations (linkage of markers) from the expected types. The test should be designed with a predetermined sensitivity and power. The number of flies in each group should reflect these defined parameters. The spontaneous mutant frequency observed in the appropriate control group will strongly influence the number of treated chromosomes that must be analysed to detect substances which show mutation rates close to those of the controls. A positive test should be confirmed by F_3 mating trials.

D. Number of replicate experiments

Replicate experiments are usually performed for each dose of the compound tested. If a chemical is a potent inducer of translocations, one experiment may be sufficient. Otherwise two or three replicate experiments should be done.

VI. DATA AND REPORT

A. Treatment of results

Data should be tabulated to show the number of translocations and the number of fertile F_1 males at each exposure for each germ cell stage sampled.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of heritable translocations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of heritable translocations or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results in the heritable translocation test in Drosophila indicate that under the test conditions the test substance causes chromosome damage in germ cells of this insect.

2. Negative results indicate that under the test conditions the test substance does not cause chromosomal damage in D. melanogaster.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. Drosphila stock used in the assay, age of insects, number of males treated, number of F_2 cultures established, number of replicate experiments;

2. test chemical vehicle, treatment and mating schedule, exposure levels, toxicity data, dose and route of exposure;
3. positive and negative (vehicle) controls;
4. historical control data, if available;
5. number of chromosomes scored;
6. criteria for scoring mutant chromosomes;
7. dose-response relationship, if applicable.

VII. REFERENCES

The following reference may be helpful in developing acceptable protocols, and provides a background of information on which this section is based. It should not be considered the only source of information on test performance, however.

1. Wurgler FE, Sobels FH, Vogel E. 1979. Drosophila as assay system for detecting genetic changes. In: Handbook of mutagenicity test procedures. Kilby BJ, Legator M, Nichols W, Ramel C, eds. Amsterdam: Elsevier/North Holland Biomedical Press, pp. 335-374.

HG-Chromo-Dom Lethal
October, 1983

RODENT DOMINANT LETHAL ASSAY

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I. PURPOSE

Dominant lethal (DL) effects cause embryonic or fetal death. Induction of a dominant lethal event after exposure to a chemical substance indicates that the substance has affected germinal tissue of the test species. Dominant lethals are generally accepted to be the result of chromosomal damage (structural and numerical anomalies) but gene mutations and toxic effects cannot be excluded.

II. DEFINITION

A dominant lethal mutation is one occurring in a germ cell which does not cause dysfunction of the gamete, but which is lethal to the fertilized egg or developing embryo.

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, triethylenemelamine, cyclophosphamide or ethyl methanesulfonate.

IV. TEST METHOD

A. Principle

Generally male animals are exposed to the test substance and mated to untreated virgin females. The various germ cell stages can be tested separately by the use of sequential mating intervals. The females are sacrificed after an appropriate period of time and the contents of the uteri are examined to determine the numbers of implants and live and dead embryos. The calculation of the dominant lethal effect is based on comparison of the live implants per female in the treated group to the live implants per female in the control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the post-implantation loss. The post-implantation loss is calculated by detertmining the ratio of dead to total implants from the treated group compared to the ratio of dead to total implants from the control group. Pre-implantation loss can be estimated on the basis of corpora lutea counts or by comapring the total implants per female in treated and control groups.

B. Description

1. Several treatment schedules are available. The most widely used requires single administration of the test substance. Other treatment schedules, such as treatment on five consecutive days, may be used if justified by the investigator.

2. Individual males are mated sequentially to virgin females at appropriate intervals. The number of matings following treatment is governed by the treatment schedule and should ensure that germ cell maturation is adequately covered. Females are sacrificed in the second half of pregnancy and the uterine contents examined to determine the total number of implants and the number of live and dead embryos.

C. Animal selection

1. Species

Rats or mice are generally used as the test species. Strains with low background dominant lethality, high pregnancy frequency and high implant numbers are recommended.

2. Age

Healthy sexually mature animals should be used.

3. Number

An adequate number of animals should be used taking into account the spontaneous variation of the biological characteristics being evaluated. The number chosen should be based on the predetermined sensitivity of detection and power of significance. For example, in a typical experiment, the number of males in each group should be sufficient to provide between 30 and 50 pregnant females per mating interval.

4. Assignment to groups

Animals should be randomized and assigned to treatment and control groups.

D. Control groups

1. Concurrent controls

Generally concurrent positive and negative (vehicle) controls should be included in each experiment. When acceptable positive control results are available from experiments conducted recently (within the last tweleve months) in the same laboratory these results can be used instead of a concurrent positive control.

2. Positive controls

Positive control substances should be used at a dose which demonstrates the test sensitivity.

E. Test chemicals

1. Vehicle

When possible, test substances should be dissolved or suspended in isotonic saline or distilled water. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles. The vehicle used should neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

2. Dose levels

Normally, three dose levels should be used. The highest dose should produce signs of toxicity (e.g. slightly reduced fertility). However, in an initial assessment of dominant lethality a single high dose may be sufficient. Nontoxic substances should be tested at 5 g/kg or, if this is not practicable, then at the highest dose attainable.

3. Route of administration

The usual routes of administration are oral or by IP injection. Other routes may be appropriate.

V. TEST PERFORMANCE

A. Individual males are mated sequentially at appropriate predetermined intervals to 1 or 2 virgin females. Females should be left with the males for at least the duration of one estrus cycle or alternatively until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug.

B. The number of matings following treatment should be governed by the treatment schedule and should ensure that germ cell maturation is adequately covered.

C. Females should be sacrificed in the second half of pregnancy and uterine contents are examined to determine the number of implants and live and dead embryos. The ovaries may be examined to determine the number of corpora lutea.

VI. DATA AND REPORT

A. Treatment of results

Data should be tabulated to show the number of males, the number of pregnant females, and the number of nonpregnant females. Results of each mating, including the identity of each male and female, should be reported individually. For each female, dose level and week of mating, the frequencies of live implants and of dead implants should be enumerated. If the data are recorded as early and late deaths, the tables should make that clear. If pre-implantation loss is estimated, it should be reported. Pre-implantation loss can be calculated as the difference between the number of corpora lutea and the number of implants or as a reduction in the average number of implants per female in comparison with control matings. Where pre-implantation loss is estimated it should be reported.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods. Differences among animals within the control and treatment groups should be considered before making comparisons between treated and control groups.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of dominant lethals. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of dominant lethals or a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. A positive DL assay suggests that under the test conditions the test substance may be genotoxic in the germ cells of the teated sex of the test species.

2. A negative result suggests that under the conditions of the test the test substance may not be genotoxic in the germ cells of the treated sex of the test species.

E. Test Report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. species, strain, age and weights of animals used, number of animals of each sex in experimental and control groups;

2. test substance, vehicle used, dose levels and rationale for dosage selection, negative (vehicle) and positive controls, experimental observations, including signs of toxicity;

3. route and duration of exposure;

4. mating schedule;

5. methods used to determine that mating has occured (where applicable);

6. time of sacrifice;

7. criteria for scoring dominant lethals;

8. dose-response relationship, if applicable.

VII. References

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Brewen JG, Payne HS, Jones KP, Preston RJ. 1975. Studies on chemically induced dominant lethality. I. The cytogenetic basis of MMS-induced dominant lethality in post-meiotic germ cells. Mutation Research 33:239-250.

2. Ehling UH, Machemer L, Buselmaier E, Dycka D, Frohberg H, Kratochvilova J, Lang R, Lorke D, Muller D, Pheh J, Rohrborn G, Roll R, Schulze-Schencking M, Wiemann H. 1978. Standard protocol for the dominant lethal test on male mice. Set up by the Work Group "Dominant lethal mutations of the ad hoc Committee Chemogenetics." Archives of Toxicology 39:173-185.

RODENT HERITABLE TRANSLOCATION ASSAYS

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
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I. PURPOSE

This test detects transmitted chromosomal damage which manifests as balanced reciprocal translocations in progeny descended from parental males treated with chemical mutagens.

II. DEFINITIONS

A. A heritable translocation is one in which distal segments of nonhomologous chromosomes are involved in a reciprocal exchange.

B. Diakinesis and metaphase I are stages of meiotic prophase scored cytologically for the presence of multivalent chromosome association characteristic of translocation carriers.

III. REFERENCE SUBSTANCES

Not applicable.

IV. TEST METHOD

A. Principle

When a balanced reciprocal translocation is induced in a parental male germ cell, the resulting progeny is a translocation heterozygote.

1. Basis for fertility screening

Male translocation heterozygotes may be completely sterile. This class consists of two types of translocations:

a. translocations between non-homologous chromosomes in which at least one of the breaks occurs close to one end of a chromosome; and,

b. those that carry multiple translocations. The majority of male translocation heterozygotes are semisterile - they carry one or (rarely) two translocations. The degree of semisterility is dependent upon the proportions of balanced and unbalanced (duplication-deficiency) gametes produced in the ejaculate as a function of meiotic segregation. Balanced and unbalanced sperm are equally capable of fertilizing an egg. Balanced sperm lead to viable progeny. Unbalanced sperm result in early embryonic lethality.

2. Basis for cytological screening

The great majority of male translocation heterozygotes can be identified cytologically through analysis of diakinesis metaphase I spermatocytes. Translocation heterozygotes are characterized by the presence of multivalent chromosome association such as a ring or chain of four chromosomes held together by chiasmata in paired homologous regions. Some translocation carriers can be identified by the presence of extra long and/or extra short chromosomes in spermatogonial and somatic cell metaphase preparations.

B. Description

Essentially, two methods have been used to screen for translocation heterozygosity; one method uses a mating sequence to identify sterile and semisterile males followed by cytological examination of suspect male individuals; the other method deletes the mating sequence altogether and all F_1 male progeny are examined cytologically for presence of translocation. In the former approach, the mating sequence serves as a screen which eliminates most fully fertile animals for cytological confirmation as translocation heterozygotes.

C. Animal selection

1. Species

The mouse is the species generally used, and is recommended.

2. Age

Healthy sexually mature animals should be used.

3. Number

   a. The number of male animals necessary is determined by the following factors:

      (1) the use of either historical or concurrent controls;
      (2) the power of the test;
      (3) the minimal rate of induction required;
      (4) whether positive controls are used and;
      (5) the level of significance desired.

4. Assignment to groups

   Animals should be randomized and assigned to treatment and control groups.

D. Control groups

1. Concurrent controls

   No concurrent positive or negative (vehicle) controls are recommended as routine parts of the heritable translocation assay. However, investigators not experienced in performing translocation testing should include a substance known to produce translocations in the assay as a positive control reference chemical.

2. Historical controls

   At the present time, historical control data must be used in tests for significance. When statistically reliable historical controls are not available negative (vehicle) controls should be used.

E. Test chemicals

1. Vehicle

   Solid and liquid test substances should be dissolved or suspended in distilled water or isotonic saline. Water insoluble chemicals may be dissolved or suspended in appropriate vehicles.

The vehicle used should neither interfere with the test chemical nor produce toxic effects. Fresh preparations of the test chemical should be employed.

2. Dose levels

At least two dose levels should be used. The highest dose level should result in toxic effects but should not produce an incidence of fatalities which would prevent a meaningful evaluation.

3. Route of administration

Acceptable routes of administration include oral, inhalation, admixture with food or water, and IP or IV injection.

V. TEST PERFORMANCE

A. Treatment and mating

The animals should be dosed with the test substance 7 days/week over a period of 35 days. After treatment, each male should be caged with 2 untreated females for a period of 1 week. At the end of 1 week, females should be separated from males and caged individually. When females give birth, the day of birth, litter size and sex of progeny are recorded. All male progeny should be weaned and all female progeny should be discarded.

B. Testing for translocation heterozygosity

When males are sexually mature, testing for translocation heterozygosity should begin. One of two methods should be used; the first method involves mating, determining those F_1 progeny which are sterile or semisterile and subsequent cytological analysis of suspect progeny; the other method does not involve mating and determining sterility or semisterility; all progeny are examined cytologically.

1. Determination of sterility or semisterility

a. Conventional method

Females are mated, usually three females for each male, and each female is killed at midpregnancy. Living and dead implantations are counted. Criteria for determining normal and semisterile males are usually established for each new strain because the number of dead implantations varies considerably among strains.

b. Sequential method

Males to be tested are caged individually with females and the majority of the presumably normal males are identified on the basis of a predetermined size of 1 or 2 litters. Breeding pens are examined daily on weekdays beginning 18 days after pairing. Young are discarded immediately after they are scored. Males that sire a litter whose size is the same as or greater than the minimum set for a translocation-free condition are discarded with their litter. If the litter size is smaller than the predetermined number, a second litter is produced with the same rule applying. Males that cannot be classified as normal after production of a second litter are tested further by the conventional method or by cytological confirmation of translocation.

2. Cytological analysis

For cytological analysis of suspected semisteriles, the air-drying technique is used. Observation of at least 2 diakinesis-metaphase 1 cells with mutivalent association constitutes the required evidence for the presence of a translocation. Sterile males are examined by one of two methods, those with testes of normal size and sperm in the epididymis are examined by the same techniques used for semisteriles. Animals with small testes are examined by squash preparations or, alternatively, by examination of mitotic metaphase preparations.

If squash preparations do not yield diakinesis-metaphase 1 cells, analysis of spermtogonia or bone marrow for the presence of unusually long or short chromosomes should be performed.

VI. DATA AND REPORT

A. Treatment of results

1. Data should be presented in tabular form and should include the number of animals at risk, the germ cell stage treated, the number of partial steriles and semisteriles (if the fertility test is used), the number of cytogenetically confirmed translocation heterozygotes (if the fertility test is used, report the number of confirmed steriles and confirmed partial steriles), the translocation rate, and either the standard error of the rate or the upper 95% confidence limit on the rate.

2. These data should be presented for both treated and control groups. Historical or concurrent controls should be specified, as well as the randomization procedure used for concurrent controls.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of heritable translocations. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of heritable translocations or a statistically significant and reproducible positive response at any one of the test points is considered non-mutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results in the heritable translocation assay indicate that under the test conditions the test substance causes heritable chromosomal damage in the test species.

2. Negative results indicate that under the test conditions the test substance does not cause heritable chromosomal damage in the test species.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. species, strain, age, weight and number of animals of each sex in each group;

2. test chemical vehicle, route and schedule of administration, toxicity data;

3. dosing regimen, doses tested and rationale for dosage selection;

4. mating schedule, number of females mated to each male;

5. the use of historical or concurrent controls;

6. screening procedure including the decision criteria used and the method by which they were determined;

7. dose-response relationship, if applicable.

VII. REFERENCES

The following reference may be helpful in developing acceptable protocols, and provides a background of information on which this section is based. It should not be considered the only source of information on test performance, however.

1. Generoso WM, Bishop JB, Goslee DG, Newell GW, Sheu C-J, von Halle E. 1980. Heritable translocation test in mice. Mutation Research 76:191-215.

HG-DNA-Damage/Repair
October, 1983

DIFFERENTIAL GROWTH INHIBITION OF REPAIR PROFICIENT AND REPAIR DEFICIENT BACTERIA: "BACTERIAL DNA DAMAGE OR REPAIR TESTS"

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

HG-DNA-Damage/Repair
October, 1983

I. PURPOSE

Bacterial DNA damage or repair tests measure DNA damage which is expressed as differential cell killing or growth inhibition of repair deficient bacteria in a set of repair proficient and deficient strains. These tests do not measure mutagenic events *per se*. They are used as an indication of the interaction of a chemical with genetic material implying the potential for genotoxicity.

II. DEFINITION

Tests for differential growth inhibition of repair proficient and repair deficient bacteria measure differences in chemically induced cell killing between wild-type strains with full repair capacity and mutant strains deficient in one or more of the enzymes which govern repair of damaged DNA.

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, chloramphenicol or methyl methanesulfonate.

IV. TEST METHOD

A. Principle

The tests detect agents that interact with cellular DNA to produce growth inhibition or killing. This interaction is recognized by specific cellular repair systems. The assays are based upon the use of paired bacterial strains that differ by the presence or absence of specific DNA repair genes. The response is expressed in the preferential inhibition of growth or the preferential killing of the DNA repair deficient strain since it is incapable of removing certain chemical lesions from its DNA.

B. Description

Several methods for performing the test have been described. Those described here are:

1. tests performed on solid medium (diffusion tests); and

2. tests performed in liquid culture (suspension tests).

C. Strain selection

1. Designation

At the present time, *Escherichia coli polA* (W3110/p3478) or *Bacillus subtilis rec* (H17/M45) pairs are recommended. Other pairs may be utilized when appropriate.

2. Preparation and storage

Stock culture preparation and storage, growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements should be performed using good microbiological techniques and should be documented.

D. Bacterial growth

Good microbiological techniques should be used to grow fresh cultures of bacteria. The phase of growth and cell density should be documented and should be adequate for the experimental design.

E. Metabolic activation

Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor supplemented postmitochondrial fraction prepared from the livers of rodents treated with enzyme inducing agents. The use of other species, tissues or techniques may also be appropriate.

F. Control groups

1. Concurrent controls

Concurrent positive, negative, and vehicle controls should be included in each assay.

2. Negative controls

The negative control should show nonpreferential growth inhibition (i.e., should affect both strains equally). Chloramphenicol is an example of a negative control.

3. Genotype specific controls

Examples of genotype specific positive controls are methyl methanesulfonate for polA strains and mitomycin C for rec strains.

4. Positive controls to ensure the efficacy of the activation system

The positive control reference substance for tests including a metabolic activation system should be selected on the basis of the type of activation system used in the test.

5. Other positive controls

Other positive control reference substances may be used.

G. Test chemicals

1. Vehicle

Test chemicals and positive and negative control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay.

2. Exposure concentrations

The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis. Because results are expressed as diameters of zones of growth inhibition in the diffusion test, it is most important that the amounts of chemical on the disc (or in the wells) are exact replicates. When appropriate, a positive response should be confirmed by testing over a narrow range of concentrations.

V. TEST PERFORMANCE

A. Diffusion assays

1. Disc diffusion assays

Disc diffusion assays may-be performed in two ways:

a. a single strain of bacteria may be added to an agar overlay or spread on the surface of the agar and the test chemical placed on a filter disc on the surface of the agar or;

b. DNA repair proficient and DNA repair deficient bacteria may be streaked in a line on the surface of the agar of the same plate and a disc saturated with test chemical placed on the surface of the agar in contact with the streaks.

2. Well diffusion assays

In well diffusion assays, bacteria may be either added to the agar overlay or spread onto the surface of the agar. A solution of the test chemical is then placed into a well in the agar.

B. Suspension assays

1. A bacterial suspension may be exposed to the test chemical and the number of surviving bacteria determined (as colony-forming units) either as a function of time of treatment or as a function of the concentration of test agent.

2. Nonturbid suspensions of bacteria may be exposed to serial dilutions of the test agent and a minimal inhibitory concentration for each strain determined, as evidenced by the presence or absence of visible growth after a period of incubation.

3. Paired bacterial suspensions (usually with some initial turbidity) may be treated with a single dose of the chemical. Positive results are indicated by a differential inhibition in the rate of increase of turbidity of the paired cultures.

C. Number of cultures

When using a plate diffusion procedure, at least two independent plates should be used at each dilution. In liquid suspension assays, at least two independent specimens for determination of the number of viable cells should be plated.

D. Incubation conditions

All plates in a given test should be incubated for the same time period. This incubation period should be for 18-24 hrs at 37 C.

VI. DATA AND REPORT

A. Treatment of results

1. Diffusion assays

Results should be expressed in diameters of zones of growth inhibition in millimeters or as areas derived therefrom as mm^2. Dose response data, if available, should be presented using the same units.

2. Liquid suspension assays

a. Survival data can be presented as dose responses, preferably as percentage of survivors or fractional survival of each strain or as a relative survival (ratio) of the two strains.

b. Results can also be expressed as the concentrations required to effect a predetermined survival rate (e.g, D_{37}, the dose permiting 37% survival). These data are derived from the survival curve. The concentration should be expressed as weight per volume, as moles, or as molarity.

c. Similarly, results can be expressed as minimal inhibitory concentration or as minimal lethal dose. The former is determined by the absence of visible growth in liquid medium and the latter is determined by plating dilutions onto semisolid media.

3. In all tests, concentrations must be given as the final concentrations during the treatment. Raw data, prior to transformation, should be provided. These should include actual quantities measured, e.g., neat numbers. For measurement of diffusion, the diameters of the discs and/or well should be indicated and the measurements should indicate whether the diameter of the discs and/or well was subtracted. Moreover, mention should be made as to whether the test chemical gave a sharp, diffuse, or double-zone of growth inhibition. If it is the latter, the investigator should indicate whether the inner or the outer zone was measured.

4. Viability data should be given as the actual plate counts with an indication of the dilution used and the volume plated or as derived titers (cells per ml). Transformed data alone in the absence of experimental data are not acceptable (i.e, ratios, differences, survival fraction).

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related preferential inhibition or killing of the repair deficient strain. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related preferential inhibition or killing of the repair deficient strain or a statistically significant and reproducible positive response at any one of the test points is considered not to interact with the genetic material of the organisms used in assay.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

DNA damage tests in bacteria do not measure DNA repair *per se* nor do they measure mutations. They measure DNA damage which is expressed as cell killing or growth inhibition. A positive result in a DNA damage test in the absence of a positive result in another system is difficult to evaluate in the absence of a better data base.

E. Test Report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. bacterial strains used;
2. phase of bacterial cell growth at time of use in the assay;
3. media composition;
4. details of both the protocol used to prepare the metabolic activation system and its use in the assay;
5. treatment protocol, including doses used and rationale for dose selection, positive and negative controls;
6. method used for determination of degree of cell kill;
7. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

2. Kada T, Sadie Y, Tutikawa K. 1972. In vitro and host-mediated "rec-assay" procedures for screening chemical mutagens; and phloxine, a mutagenic red dye detected. Mutation Research 16:165-174.

3. Leifer Z, Kada T, Mandel M, Zeiger E, Stafford R, Rosenkranz HS. 1981. An evaluation of bacterial DNA repair tests for predicting genotoxicity and carcinogenicity: a report of the U.S. EPA's Gene-Tox Program. Mutation Research 87:211-297.

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HG-DNA-Unsched Syn
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UNSCHEDULED DNA SYNTHESIS IN MAMMALIAN CELLS IN CULTURE

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I. PURPOSE

Unscheduled DNA synthesis (UDS) in mammalian cells in culture measures the repair of DNA damage induced by a variety of agents including chemicals, radiation and viruses. UDS may be measured in both in vitro and in vivo systems.

II. DEFINITION

In this guideline, unscheduled DNA synthesis in mammalian cells in culture is defined as the incorporation of tritium labelled thymidine (^{3}H-TdR) into the DNA of cells which are not in the S phase of the cell cycle.

III. REFERENCE SUBSTANCES

These may include, but need not be limited to, 7,12-dimethylbenzanthracene, 2-acetylaminofluorene, 4-nitroquinoline oxide or N-dimethyl-nitrosamine.

IV. TEST METHOD

A. Principle

Mammalian cells in culture, either primary cultures of rodent hepatocytes or established cell lines, are exposed to the test agent. Established cell lines are treated both with and without metabolic activation. UDS is measured by the uptake of ^{3}H-TdR into the DNA of non-S phase cells. Uptake may be determined by autoradiography or by liquid scintillation counting (LSC) of DNA from treated cells.

B. Description

1. Autoradiography

For autoradiography, coverslip cultures of cells are exposed to test chemical in medium containing ^{3}H-TdR. At the end of the treatment period, cells are fixed, dipped in autoradiographic emulsion, and exposed at 4 C. At the end of the exposure period, cells are stained and labeled nuclei are counted either manually or with an electronic counter. Established cell lines should be treated both with and without metabolic activation.

2. LSC determinations

For LSC determinations of UDS, confluent cultures of cells are treated with test chemical both with and without metabolic activation. At the end of the exposure period, DNA is extracted from the treated cells. Total DNA content is determined and extent of ^{3}H-TdR incorporation is determined by scintillation counting.

C. Cells

1. Type of cells used in the assay

A variety of cell lines or primary cell cultures, including human cells may be used in the assay.

Established cell lines should be checked for *Mycoplasma* contamination and may be periodically checked for karyotype stability.

2. Cell growth and maintenance

Appropriate culture media and incubation conditions (culture vessels CO_2 concentration, temperature and humidity should be used.

D. Metabolic activation

1. A metabolic activation system is not used with primary cultures of rodent hepatocytes.

2. Established cell lines should be exposed to test substance both in the presence and absence of an appropriate metabolic activation system.

E. Control groups

Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation as appropriate should be included in each experiment.

F. Test chemicals

1. Vehicle

Test chemicals and positive control reference substances may be prepared in culture media or dissolved or suspended in appropriate vehicles prior to treatment of the cells. Final concentration of the vehicle should not interfere with cell viability or growth rate.

2. Exposure concentrations

Multiple concentrations of test substance, based upon cytotoxicity, and over a range adequate to define the response should be used. For cytotoxic chemicals, the first dose to elicit a cytotoxic response in a preliminary assay should be the highest dose tested. Relatively insoluble compounds should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

V. TEST PERFORMANCE

A. Primary cultures of rodent hepatocytes

Freshly isolated rodent hepatocytes should be treated with chemical in medium containing ^{3}H-TdR. At the end of the treatment period, cells should be drained of medium, rinsed, fixed, dried and attached to microscope slides. Slides should be dipped in autoradiographic emulsion, exposed at 4 C for an appropriate length of time, developed, stained and counted.

B. Established cell lines

1. Autoradiographic techniques

The techniques for treatment of established cell lines are the same as those for primary cultures of rodent hepatocytes except that cells must not enter S phase prior to treatment. Entry of cells into S phase may be blocked by several methods (e.g. by growth in medium deficient in arginine or low in serum or by treatment with chemical agents such as hydroxyurea). Tests should be done both in the presence and absence of a metabolic activation system.

2. LSC measurement of UDS

Prior to treatment with test agent, entry of cells into S phase should be blocked as described above. Cells should be exposed to the test chemical in medium containing ^{3}H-TdR. At the end of the incubation period, DNA should be extracted from the cells by hydrolysis with perchloroacetic acid or by other acceptable methods. One aliquot of DNA is used to determine total DNA content; a second aliquot is used to measure the extent of ^{3}H-TdR incorporation.

C. Acceptable background frequencies

1. Autoradiographic determinations

Net incorporation of ^{3}HTdR into the nucleus of solvent treated control cultures should be less than 1.

2. LSC determinations

Historical background incorporation rates of ^{3}H-TdR into untreated established cell lines should be established for each laboratory.

D. Number of cells counted

A minimum of 50 cells per culture should be counted for autoradiographic UDS determinations. Slides should be coded before being counted. Several widely separated random fields should be counted on each slide. Cytoplasm adjacent to the nuclear areas should be counted to determine spontaneous background.

E. Number of cultures

Six independent cultures at each concentration and control should be used in LSC UDS determinations.

VI. DATA AND REPORT

A. Treatment of results

1. Autoradiographic determinations

For autoradiographic determinations, once untransformed data are recorded, background counts should be subtracted to give the correct nuclear grain count. Values should be reported as net grains per nucleus. Mean, median and mode may be used to describe the distribution of net grains per nucleus.

2. LSC determinations

For LSC determinations, ^{3}H-TdR incorporation should be reported as dpm/ug DNA. Average dpm/ug DNA with standard deviation or standard error of the mean may be used to describe distribution of incorporation in these studies.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the incorporation of ^{3}H-TdR into treated cells. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related increase in the incorporation of ^{3}H-TdR into treated cells or a statistically significant and reproducible positive response at any one of the test points is considered not to induce UDS in the test system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results in the UDS assay indicate that under the test conditions the test substance may induce DNA damage in cultured mammalian somatic cells.

2. Negative results indicate that under the test conditions the test substance does not induce DNA damage in cultured mammalian somatic cells.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. cells used, density and passage number at time of treatment, number of cell cultures;
2. methods use for maintenance of cell cultures including medium, temperature and CO_2 concentration;
3. test chemical vehicle, concentration and rationale for selection of concentrations used in the assay;
4. details of both the protocol used preparation of the metabolic activation system and its used in the assay;
5. treatment protocol;
6. positive and negative controls;
7. protocol used for autoradiography;
8. details of the method used to block entry of cells into S phase;
9. details of the methods used for DNA extraction and determination of total DNA content in LSC determinations;
10. historical background incorporation rates of ^{3}H-TdR in untreated cell lines;
11. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

2. Rasmussen RE, Painter RB. 1966. Radiation-stimulated DNA synthesis in cultured mammalian cells. J Cell Biol 29:11-19.

3. Stich HF, San PPS, Lam KJ, Koropatnick DJ, Lo LW, Laishes BA. 1976. DNA fragmentation and DNA repair as an in vitro and in vivo assay for chemical procarcinogens, carcinogens and carcinogenic nitrosation products. In: Screening tests in chemical carcinogenesis. Bartsch H, Tomatis L, eds. Lyon: IARC Scientific Publications, No. 12, pp. 617-636.

4. Williams GM. 1976. Carcinogen-induced DNA repair in primary rat liver cell cultures: a possible screen for chemical carcinogens. Cancer Letters 1:231-236.

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HG-DNA-Gene Conversion
October, 1983

MITOTIC GENE CONVERSION IN *SACCHAROMYCES CEREVISIAE*

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HG-DNA-Gene Conversion
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I. PURPOSE

The mitotic gene conversion assay in the yeast, Saccharomyces cerevisiae, measures the conversion of differentially inactive alleles to wild-type alleles by mutagenic agents. Heteroallelic diploid yeast strains carry two different inactive alleles of the same gene locus. The presence of these alleles causes a nutritional requirement, e.g. these heteroallelic diploids grow only in medium supplemented with a specific nutrient such as tryptophan. When gene conversion occurs, a fully active wild-type phenotype is produced from these inactive alleles through intragenic recombination. These wild-type colonies grow on a medium lacking the specific nutritional requirement (selective medium).

II. DEFINITIONS

A. Mitotic gene conversion is detected by the change of inactive alleles of the same gene to wild-type alleles through intragenic recombination in mitotic cells.

B. Heteroallelic diploids are diploid strains of yeast carrying two different, inactive alleles of the same gene locus causing a nutritional requirement.

III. REFERENCE SUBSTANCES

These may include but need not be limited to, hydrazine sulfate or 2-acetylaminofluorene.

IV. TEST METHOD

A. Principle

The method is based on the fact that heteroallelic diploid yeast strains carry two inactive alleles of the same gene locus making them dependent on a specific nutritional requirement (e.g. tryptophan) for their survival. Treatment of such strains with mutagenic agents can cause conversion of these alleles back to the wild-type condition which allows growth on a medium lacking the required nutrient (selective medium).

B. Description

Heteroallelic diploid strains such as D7, requiring a specific nutrient in the medium, are treated with test chemical with and without metabolic activation and plated on a selective medium lacking the required nutrient. The wild-type colonies that grow on the selective medium as a result of gene conversion are scored.

C. Strain selection

1. Designation

At the present time, S. cerevisiae strain D7 is recommended for use in this assay. The use of other strains may also be appropriate.

2. Preparation and storage

Stock culture preparation and storage, growth requirements, method of strain identification and demonstration of appropriate phenotypic requirements requirements should be performed using good microbiological techniques and should be documented.

3. Media

YEP glucose medium enriched with the appropriate growth factors may be used for cell growth and maintenance. Other media may also be appropriate.

D. Selection of cultures

Cells should be grown with aeration in liquid medium enriched with growth factors to early stationary phase. Cells should then be seeded on selective medium to determine the rate of spontaneous conversion. Cultures with a high rate of spontaneous conversion should be discarded.

E. Metabolic activation

Cells should be exposed to test chemical both in the presence and absence of an appropriate metabolic activation system.

F. Control groups

Concurrent positive and negative (untreated and/or vehicle) controls both with and without metabolic activation should be included in each expermient.

G. Test chemicals

1. Vehicle

Test chemicals and positive control reference substances should be dissolved in an appropriate vehicle and then further diluted in vehicle for use in the assay. Dimethylsulfoxide should be avoided as a vehicle.

2. Exposure concentrations

a. The test should initially be performed over a broad range of concentrations. Among the criteria to be taken into consideration for determining the upper limits of test chemical concentration are cytotoxicity and solubility. Cytotoxicity of the test chemical may be altered in the presence of metabolic activation systems. For cytotoxic chemicals, the highest dose tested should not reduce survival to less than 10% of that seen in the untreated control cultures. Relatively insoluble chemicals should be tested up to the limits of solubility. For freely soluble nontoxic chemicals, the upper test chemical concentration should be determined on a case by case basis.

b. When appropriate, a positive response should be confirmed by using a narrow range of concentrations.

V. TEST PERFORMANCE

A. Treatment

Cultures should be treated in liquid suspension. Resting cells should be treated in buffer; growing cells should be treated in a synthetic medium. Cultures with low spontaneous convertant frequencies should be centrifuged, washed and resuspended in liquid at the appropriate density. Cells should be exposed to test chemical both in the presence and absence of a metabolic activation system. Independent tubes should be treated for each concentration. At the end of the treatment period, cells should be centrifuged, washed and resuspended in distilled water prior to plating on selective medium for convertant selection and on complete medium to determine survival. At the end of the incubation period, plates should be scored for survival and the presence of convertant colonies.

B. Number of cultures

At least six individual plates per treatment concentration and control should be used.

C. Incubation conditions

All plates in a given experiment should be incubated for the same time period. This incubation period may be from 4-6 days at 28 C.

VI. DATA AND REPORT

A. Treatment of results

Individual plate counts for test substance and control should be presented for both convertants and survivors. The mean number of colonies per plate and standard deviation should also be presented. Data should be presented in tabular form indicating numbers of viable and convertant colonies scored, survival frequency and convertant frequencies for each treatment and control culture. Conversion frequencies should be expressed as number of convertants per number of survivors. Sufficient detail should be provided for verification of survival and convertant frequencies.

B. Statistical evaluation

Data should be evaluated by appropriate statistical methods.

C. Interpretation of results

1. There are several criteria for determining a positive result, one of which is a statistically significant dose-related increase in the number of gene convertants. Another criterion may be based upon detection of a reproducible and statistically significant positive response for at least one of the test points.

2. A test substance which does not produce either a statistically significant dose-related increase in the number of gene conversions nor a statistically significant and reproducible positive response at any one of the test points is considered nonmutagenic in this system.

3. Both biological and statistical significance should be considered together in the evaluation.

D. Test evaluation

1. Positive results in this assay indicate that under the test conditions the test chemical causes mitotic gene conversion in the yeast S. cerevisiae.

2. Negative results indicate that under the test conditions the test chemical does not cause mitotic gene conversion in S. cerevisiae.

E. Test report

In addition to the reporting recommendations as specified in the EPA Good Laboratory Practices Standards (Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations) the following specific information should be reported:

1. strain of organism used in the assay;

2. test chemical vehicle, doses used and rationale for dosage selection;

3. method used to select cultures;

4. treatment protocol including cell density at treatment and length of exposure to test substance;

5. details of both the protocol used to prepare the metabolic activation system and its use in the assay;

6. incubation times and temperatures;

7. dose-response relationship, if applicable.

VII. REFERENCES

The following references may be helpful in developing acceptable protocols, and provide a background of information on which this section is based. They should not be considered the only source of information on test performance, however.

1. Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

2. Callen DF, Philpot RM. 1975. Cytochrome P-450 and the activation of promutagens in Saccharomyces cerevisiae. Mutation Research 45:309-324.

3. Zimmermann FK. 1979. Procedures used in the induction of mitotic recombination and mutation in the yeast Saccharomyces cerevisiae. In: Handbook of mutagenicity test procedures. Kilby BJ, Legator M, Nicols W, Ramel C, eds. Amsterdam: Elsevier/North Holland Biomedical Press, pp. 119-134.

4. Zimmermann FK, Kern R, Rosenberger H. 1975. A yeast strain for simultaneous detection of induced mitotic crossing over, mitotic gene conversion and reverse mutation. Mutation Research 28:381-388.

HG-Neuro-Path
October, 1983

NEUROPATHOLOGY

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I. PURPOSE

The techniques in this guideline are designed to develop data on morphologic changes in the nervous system for chemical substances and mixtures subject to such testing under the Toxic Substances Control Act. The data will detect and characterize morphologic changes, if and when they occur, and determine a no-effect level for such changes. Neuropathological evaluation should be complemented by other neurotoxicity studies, e.g. behavioral and neurophysiological studies. Neuropathological evaluation may be done following acute, subchronic or chronic exposure.

II. DEFINITIONS

Neurotoxicity or a neurotoxic effect is an adverse change in the structure or function of the nervous system following exposure to a chemical agent.

III. PRINCIPLE OF THE TEST METHOD

The test substance is administered to several groups of experimental animals, one dose being used per group. The animals are sacrificed and tissues in the nervous system are examined grossly and prepared for microscopic examination. Starting with the highest dosage level, tissues are examined under the light microscope for morphologic changes, until a no effect level is determined. In cases where light microscopy has revealed neuropathology, the no effect level may be confirmed by electron microscopy.

IV. TEST PROCEDURE

A. Animal Selection.

1. Species and Strain.

Testing should be performed in the species being used in other tests for neurotoxicity. This will generally be the laboratory rat. The choice of species shall take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies, the potential for combined studies, and the availability of other toxicity data for the species.

2. Age.

Animals shall be young adults (150-200 gm for rats) at the start of exposure.

3. Sex.

Both sexes should be used unless it is demonstrated that one sex is refractory to the effects.

B. Number of Animals.

A minimum of six animals per group shall be used. The tissues from each animal shall be examined separately. It is recommended that ten animals per group be used.

C. Control Groups.

1. A concurrent control group(s) is (are) required. This group must be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the vehicle used has a known or potential toxic property, both untreated and vehicle control groups are required.

2. A satellite group of animals may be treated with the high level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

D. Dose Levels and Dose Selection.

At least 3 doses, equally spaced on a log scale (e.g., 1/2 log units) over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve. The highest dose shall produce 1) clear behavioral effects or 2) life-threatening toxicity. The data from the lower doses must show either 1) graded dose-dependent effects at 2 dose levels or 2) no effects at 2 dose levels, respectively.

E. Duration of Testing.

The exposure duration will be specified in the test rule. This will generally be 90 days exposure.

F. Route of Administration.

The test substance shall be administered by a route specified in the test rule. This will generally be the route most closely approximating the route of human exposure. The exposure protocol shall conform to that outlined in the appropriate acute or subchronic toxicity guideline.

G. Combined Protocol.

The tests described herein may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination.

H. Study Conduct.

1. Observation of Animals.

All toxicological (e.g. weight loss) and neurological signs (e.g. motor disturbance) shall be recorded frequently enough to observe any abnormality, and not less than weekly.

2. Sacrifice of Animals.

a. General.

The goal of the techniques outlined for sacrifice of animals and preparation of tissues is preservation of tissue morphology to simulate the living state of the cell.

b. Perfusion Technique.

Animals shall be perfused *in situ* by a generally recognized technique. For fixation suitable for light or electron microscopy, saline solution followed by buffered 2.5% glutaraldehyde or buffered 4.0% paraformaldehyde, is recommended. While some minor modifications or variations in procedures are used in different laboratories, a detailed and standard procedure for vascular perfusion may be found in the text by Zeman and Innes (1963), Hayat (1970) and by Spencer and Schaumburg (1980). A more sophisticated technique is described by Palay and Chan-Palay (1974).

c. Removal of Brain and Cord.

After perfusion, the bony structure (cranium and vertebral column) should be exposed. Animals should then be stored in fixative-filled bags at 4°C for 8-12 hours. The cranium and vertebral column shall be removed carefully by trained technicians without physical damage of the brain and cord. Detailed dissection procedures may be found in the text by Palay and Chan-Palay (1974). After removal, simple measurement of the size (length and width) and weight of the whole brain (cerebrum, cerebellum, pons-medulla) should be made. Any abnormal coloration or discoloration of the brain and cord should also be noted and recorded.

d. Sampling.

Unless a given test rule specifies otherwise, cross-sections of the following areas shall be examined: the forebrain, the center of the cerebrum, the midbrain, the cerebellum and pons, and the medulla oblongata; the spinal cord at cervical and lumbar swellings (C_3-C_6 and L_1-L_4); Gasserian ganglia, dorsal root ganglia (C_3-C_6, L_1-L_4), dorsal and ventral root fibers (C_3-C_6, L_1-L_4), proximal sciatic nerve (mid-thigh and sciatic notch), sural nerve (at knee), and tibial nerve (at knee). Other sites and tissue elements (e.g. gastrocnemius muscle) should be examined if deemed necessary. Any observable gross changes shall be recorded.

3. Specimen Storage.

Tissue samples from both the central and peripheral nervous system shall be further immersion fixed and stored in appropriate fixative (e.g. 10% buffered formalin for light microscopy; 2.5% buffered gluteraldehyde or 4.0% buffered paraformaldehyde for electron microscopy) for future examination. The volume of fixative versus the volume of tissues in a specimen jar shall be no less than 25:1. All stored tissues should be washed with buffer for at least 2 hours prior to further tissue processing.

4. Histopathology Examination.

a. Fixation.

Tissue specimens stored in 10% buffered formalin may be used for this purpose. All tissues must be immersion fixed in fixative for at least 48 hours prior to further tissue processing.

b. Dehydration.

All tissue specimens should be washed for at least one hour with water or buffer, prior to dehydration. (A longer washing time is needed if the specimens have been stored in fixative for a prolonged period of time). Dehydration can be performed with increasing concentration of graded ethanols up to absolute alcohol.

c. Clearing and Embedding.

After dehydration, tissue specimens shall be cleared with xylene and embedded in paraffin or paraplast. Multiple tissue specimens (e.g. brain, cord, ganglia) may be embedded together in one single block for sectioning. All tissue blocks shall be labelled showing at least the experiment number, animal number, and specimens embedded.

d. Sectioning.

Tissue sections, 5-6 microns in thickness, shall be prepared from the tissue blocks and mounted on standard glass slides. It is recommended that several additional sections be made from each block at this time for possible future needs for special stainings. All tissue blocks and slides shall be filed and stored in properly labelled files or boxes.

e. Histopathological Techniques.

Although the information available for a given chemical substance may dictate test-rule specific changes, the following general testing sequence is proposed for gathering histopathological data:

(1) General Staining.

A general staining procedure shall be performed on all tissue specimens in the highest treatment group. Hematoxylin and eosin (H&E) shall be used for this purpose. The staining shall be differentiated properly to achieve bluish nuclei with pinkish background.

(2) Special Stains.

Based on the results of the general staining, selected sites and cellular components shall be further evaluated by the use of specific techniques. If H&E screening does not provide such information, a battery of stains shall be used to assess the following components in all appropriate required samples: neuronal body (e.g. Einarson's gallocyanin), axon (e.g. Bodian), myelin sheath (e.g. Kluver's Luxol Fast Blue) and neurofibrils (e.g. Bielchosky). In addition, peripheral nerve fiber teasing shall be used. Detailed staining methodology is available in standard histotechnological manuals such as AFIP (1968), Ralis et al. (1973), and Chang (1979). The nerve fiber teasing technique is discussed in Spencer and Schaumberg (1980). A section of normal tissue shall be included in each staining to assure that adequate staining has occurred. Any changes shall be noted and representative photographs shall be taken. If a lesion(s) is observed, the special techniques shall be repeated in the next lower treatment group until no further lesion is detectable.

(3) Alternative Technique.

If the anatomical locus of expected neuro-pathology is well-defined, epoxy-embedded sections stained with toluidine blue may be used for small sized tissue samples. This technique obviates the need for special stains for cellular components. Detailed methodology is available in Spencer and Schaumberg (1980).

(4) Electron Microscopy.

Based on the results of light microscopic evaluation, specific tissue sites which reveal a lesion(s) shall be further evaluated by electron microscopy in the highest treatment group which does not reveal any light microscopic lesion. If a lesion is observed, the next lower treatment group shall be evaluated until no significant lesion is found. Detailed methodology is available in Hayat (1970).

f. Examination.

(1) General.

All stained microscopic slides shall be examined with a standard research microscope. Examples of cellular alterations (e.g., neuronal vacuolation, degeneration, and necrosis) and tissue changes (e.g., gliosis, leukocytic infiltration, and cystic formation) shall be recorded and photographed.

(2) Electron Microscopy.

Since the size of the tissue samples that can be examined is very small, at least 3-4 tissue blocks from each sampling site must be examined. Tissue sections must be examined with a transmission electron microscope. Three main categories of structural changes must be considered:

(a) Neuronal body.

The shape and position of the nucleus and nucleolus as well as any change in the chromatin patterns shall be noted. Within the neuronal cytoplasm, cytoplasmic organelles such as mitochondria, lysosomes, neurotubules, neurofilaments, microfilaments, endoplasmic reticulum and polyribosomes (Nissl substance), Golgi complex, and secretory granules shall be examined.

(b) Neuronal processes.

The structual integrity or alterations of dendrites, axons (myelinated and unmyelinated), myelin sheaths, and synapses shall be noted.

(c) Supporting cells.

Attention must also be paid to the number and structural integrity of the neuroglial elements (oligodendrocytes, astrocytes, and microglia) of the central nervous system, and the Schwann cells, satellite cells, and capsule cells of the peripheral nervous system. Any changes in the endothelial cells and ependymal lining cells shall also be noted whenever possible. The nature, severity, and frequency of each type of lesion in each specimen must be recorded. Representative lesions must be photographed and labeled appropriately.

V. DATA COLLECTION, REPORTING, AND EVALUATION

In addition to information meeting the requirements stated in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40 Code of Regulations], the following specific information should be reported:

A. Description of Test System and Test Methods

A description of the general design of the experiment should be provided. This should include a short justification explaining any decisions where professional judgement is involved such as fixation technique and choice of stains.

B. Results

All observations shall be recorded and arranged by test groups. This data may be presented in the following recommended format:

1. Description of Signs and Lesions for Each Animal.

   For each animal, data must be submitted showing its identification (animal number, treatment, dose, duration), neurologic signs, location(s), nature of, frequency, and severity of lesion(s). A commonly-used scale such as 1+, 2+, 3+, and 4+ for degree of severity ranging from very slight to extensive may be used. Any diagnoses derived from neurologic signs and lesions including naturally occurring diseases or conditions, should also be recorded.

2. Counts and Incidence of Lesions, by Test Group.

   Data shall be tabulated to show: a. The number of animals used in each group, the number of animals displaying specific neurologic signs, and the number of animals in which any lesion was found; b. The number of animals affected by each different type of lesion, the average grade of each type of lesion, and the frequency of each different type and/or location of lesion.

3. Evaluation of Data.

   An evaluation of the data based on gross necropsy findings and microscopic pathology observations shall be made and supplied. The evaluation shall include the relationship, if any, between the animal's exposure to the test substance and the frequency and severity of the lesions observed.

The evaluation of dose-response, if existent, for various groups shall be given, and a description of statistical method must be presented. The evaluation of neuropathology data should include, where applicable, an assessment in conjunction with other neurotoxicity studies performed (eg. electrophysiological, behavioral, neurochemical).

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NEUROPATHOLOGY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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TABLE OF CONTENTS

PAGE

I. NEUROPATHOLOGY IN TOXICOLOGY 1

- A. Nervous system as a target organ 1
- B. Neuropathology (morphology) as a tool in detecting neurotoxicity 2
- C. Basic objective of the test standard in neuropathology 2

II. RATIONALES FOR STUDY DESIGN 2

- A. Species 2
- B. Sex and age 3
- C. Number of animals 3
- D. Dose and duration of testing 3
- E. Route of exposure 3
- F. Technical personnel and pathologist requirements 4

III. RATIONALES FOR STUDY CONDUCT 4

- A. Proper techniques in animal sacrifice and tissue handling 4
- B. Vascular perfusion 5
- C. Fixative of choice 6
- D. Gross examination of brain and cord 6
  - 1. Brain weight and size 6
  - 2. Gross appearance of the brain and cord. 7
- E. Specimen storage 7
- F. General histopathological evaluation 8
  - 1. Tissue sampling 8
  - 2. Tissue processing 8
  - 3. Paraffin/hematoxylin-eosin (H & E) or epoxy-toluidine blue technique as a general screening method 9
  - 4. Data collection, evaluation, and reporting 10

IV. SPECIFIC DETECTION AND EVALUATION TECHNIQUES ... 10

- A. Need for special techniques 10
- B. General objective of the special techniques 11
- C. Methods selected for specific evaluation of neural changes 11

D. When to perform 12
E. Test scheme 13
F. Economy of the special evaluation methods . 16

V. ELECTRON MICROSCOPY 17

A. Objective 17
B. Limitations and advantages 17
C. When to perform 18
D. Test scheme 18
E. Elements to be examined 19

REFERENCES 20

I. NEUROPATHOLOGY IN TOXICOLOGY

A. Nervous System as a Target Organ

Many chemical compounds such as acetylethyl tetramethyl tetralin (AETT), acrylamide, heavy metals, carbon monoxide, organophosphorous compounds, to name a few, are found to have toxic effects on the nervous system. Because of the unique structure and functional properties of the nervous system, neurotoxicology is becoming a specialized area of investigation (Spencer and Schaumberg 1980).

Since the nervous system either dominates or influences most functions of the organism and is not, in a general sense, capable of full regeneration after severe injury, any toxic assault on the nervous system may create a significant and long-lasting impact on the health and function of the organism. Therefore, the identification and understanding of neurotoxicants are of primary importance.

Toxic substances can affect various aspects of the nervous system (biochemical, physiological, morphological, and behavioral), inducing changes in sensory, motor, cognitive or emotional function. These various disciplines may be used to detect and evaluate the impact of substances on the nervous system. The guideline for neuropathology focuses on selected procedures for detection and evaluation of morphological changes in the nervous system.

B. Neuropathology (Morphology) as a Tool in Detecting Neurotoxicity

Cells in the nervous system, like cells in other systems, can function properly only upon total structural integrity. Unlike most other cells in the body of the organisms, nervous-system cells exhibit a highly specialized architecture: neurons, myelinating cells (oligodendrocytes and Schwann cells), and astrocytes each possess one or more long cellular processes which depend for their maintenance on synthetic activities carried out at a remote site in the cell and the transport of nutrients over long distances. The specialized architectural design of nervous system cells thus provides much greater vulnerability to toxic attack than cells of other systems (Spencer and Schaumburg 1980).

Because the nervous system has a functional reserve and exhibits a certain degree of plasticity, functional disturbance in the form of neurological signs or behavioral changes may not be manifest until a significant amount of structural damage has been incurred. Depletion of the structural reserve of the nervous system may make the organism more vulnerable to subsequent toxic attack, or to the effects of abnormal metabolism or aging. Therefore, early morphological alterations in the nervous system, with or without functional deficits, represent a significant and sensitive indication of neural damage.

C. Basic Objective of the Guideline in Neuropathology

The basic goal of the techniques developed for evaluation of morphological changes is to preserve the tissue morphology to simulate the living state of the cell. The objective of this guideline is to present a standard approach for sensitive detection and systematic evaluation of morphological changes in various components of the nervous tissues and cells: neuronal cell body, axons, dendrites, myelin sheaths, nerve fibers, and subcellular organelles.

II. Rationales for Study Design

A. Species

The species being studied for functional neurotoxicity assessment should also be evaluated for morphological changes. Morphological data generated in this guideline, therefore, can then be correlated with data generated in other studies. Rodents will generally be the species of choice because (a) They have been used as animal models for the investigations of the local or systemic toxicities of chemical substances. The information generated on the pharmacokinetics (e.g., distribution, excretion, etc.) and target organ toxicities (e.g., on kidney, liver, etc.) of the chemicals may enhance the understanding of the toxic impacts on the nervous system. (b) Pure bred (genetically known) strains are available, which enhances the reproducibility of the experiments. The comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance are also important factors for consideration.

B. Sex and Age

Toxic susceptibility or sensitivity is known to vary with age and sex of the animals. Young adults should be used because of their fully matured nervous system (unless the experiment is designed to study toxic impacts on a system which may decline with age). If scientific judgement deems data from males and females is warranted, both should be used.

C. Number of Animals

A minimum of six animals per group is recommended because, if examined separately, this will provide enough tissues for acceptable evaluation.

D. Dose and Duration of Testing

At least three dosage groups (high, medium, and low) should be tested. It is recommended that the high dose selected should not be acutely lethal to the animals (neuropathology usually takes time to develop) but should induce some observable neurological signs which may be helpful in determining the location(s) of the lesion(s). The low dose level selected hopefully will not induce overt signs for about 90 days. This "no observed effect level" is important for the evaluation of the risk of long-term, low dose exposure to the substance. Recommendations in this standard are also consistent with those recommended in the federal guidelines for other neurotoxicity studies. Since neuropathology is an integral part of the overall neurotoxicology study, the general scheme for animal treatment (dose levels, duration of exposure, etc.) should be in line with those recommended for other aspects of neurotoxicological study so that data generated can support, complement, and supplement other studies.

E. Route of Exposure

The route of exposure used should be consistent across neuro- and other toxicity studies to facilitate comparison of results. Different routes of exposure will result in different rates of absorption and excretion of the test substance and thus influence the toxic effect of the substance. All routes of exposure (inhalation, injection, intubation, etc.) have been used by different investigators to induce neurotoxicity. However, the selection of the method of administration should be dictated by the nature of the test substance and the metabolism (if known) of the test substance as well as the route of human exposure.

6. Technical Personnel and Pathologist Requirements

The general requirements for personnel are discussed in the EPA Good Laboratory Practice Standards [Subpart B, Part 792, Chapter I of Title 40, Code of Federal Regulations]. It is further recommended in this test guideline that the pathologist in charge of the project not only should have been trained basically in pathology (M.D., D.V.M., D.O. or Ph.D. in Experimental Pathology), but also should have special training or experience in neuropathology. This is recommended because neuropathology is an extremely specialized field and often has its own technical "language." A lack of formal training or experience in this area will severely limit the investigator's ability in directing the experiments and in interpreting the findings. For projects that involve electron microscopy, the pathologist should also be well aware of the techniques of electron microscopy and well-trained in the interpretation and data analysis of ultrastructures of nervous tissues. A general electron microscopist, cytologist, or neuroanatomist will not be able to manage the complexity of neuropathology at the ultrastructural level.

III. RATIONALES FOR THE STUDY CONDUCT

A. Proper Techniques in Animal Sacrifice and Tissue Handling

Improper sacrifice and tissue handling techniques frequently produce irreversible artifacts in the nervous tissues. Therefore, all techniques should be performed by trained laboratory personnel who are experienced in this area of research. Although there are various techniques which are equally acceptable for neuromorphological research, they all share similar basic principles. The techniques and methodologies which are recommended in this test standard represent those proven to be reliable, reproducible, cost efficient, and easy to perform.

The purpose of anatomic pathology is to make morphological evaluation of disease (pathological) states of the cells and tissues. Thus, the same general principles and techniques for morphological research as those employed by anatomists should be followed to ensure proper morphological preservation.

B. Vascular Perfusion

Nervous tissues, particularly those of the CNS, are subjected to rapid anoxic and autolytic changes after death of the animals. Rapid fixation by means of vascular perfusion will help to prevent such undesirable changes. Perfusion fixation also serves to harden the nervous tissue and reduce the chances of artifact occurrence during brain and cord removal. During the past decade, procedures for proper fixation and preservation of nervous tissues have undergone considerable changes and modification. For good morphological preservation, all animals should be perfused for both light and electron microscopy. The available data indicate that the quality of preservation of the fine structure is affected not only by the characteristics of the fixative, but also by the method of applying the fixative to the tissue. Comparative studies have shown that the rate of penetration of a fixative is influenced by various conditions prevalent before and during fixation. It appears that under *in vivo* conditions, the rate and depth of fixation are increased by perfusion fixation. For example, rat kidneys fixed *in vivo* by flooding with 1% osmium tetroxide were penetrated at a rate of about 20 um per minute (Maunsbach et al. 1962), whereas similar tissues fixed *in vitro* were penetrated at a rate of only about 8 um per minute (Rhodin, 1954). The above tissues fixed *in vivo* also showed good fixation to a depth of 100 to 200 um compared with only 30 to 40 um depth achieved through *in vitro* fixation. This increased rate of penetration is probably related to an increased tubular flow of the fixative in the living kidneys.

The method employed in *in vivo* fixation is perfusion of a suitable fixing agent through the vascular channels. This method essentially involves a rapid and uniform penetration of a fixative into all parts of the tissue, prior to any injury to the tissue by anoxia or direct handling. Fixation by perfusion is expected to stabilize the tissue against diffusion and translocation of cellular substances, which may occur during fixation by immersion. The application of this method is thus especially useful for tissues that are exceptionally sensitive to the effects of oxygen deprivation and physical handling. Neural tissues, especially the central nervous system are satisfactorily fixed only by perfusion methods. Under optimal fixation conditions, an artifact-free nervous system tissue can be obtained.

C. Fixative of Choice

Although 10% buffered formalin is an excellent general fixative for light microscopy, it has a very high aldehyde content and hardens the tissues too much for electron microscopy. Phosphate-buffered 2.5% glutaraldehyde or 4.0% paraformaldehyde having an osmolality of about 600-800 mOsM at pH 7.4 is excellent for perfusion of brain tissues. Furthermore, tissues perfused with these fixatives can be subjected to further immersion fixation in 10% buffered formalin or in the same fixative; thus permitting both light and electron microscopy investigation. A mixture of paraformaldehyde and glutaraldehyde (modified Karnovsky) is also an excellent perfusate and fixative for the nervous system. However, it requires more labor and cost in its preparation and does not pose a significant advantage over glutaraldehyde or paraformaldehyde alone.

D. Gross Examination of Brain and Cord

1. Brain Weight and Size.

An edematous (swelling) condition (e.g., acute lead poisoning) may be induced under some neurotoxic conditions. Simple weighing and measuring of the removed brain may generate this data. Such procedures can be done within one minute and are of no cost to the investigator. However, the data collected may be of great value.

2. Gross Appearance of the Brain and Cord.

Simple observation of the gross appearance of the brain and cord may yield invaluable pathological information, such as petechial hemorrhage (e.g., in carbon monoxide poisoning) or area of necrosis. This observation can be made during brain/cord removal and cutting and imposes no significant time or cost to the investigator. The brain should be cut at various levels for both gross and light microscopic examination. Frontal plane cuttings which can reveal various internal structures of the brain on both hemispheres are recommended.

The cervical and lumbar segments of the cord (C_3-C_6, L_1-L_4) are selected for special attention because these are the most prominent areas of the cord controlling the input/output of the upper and lower extremities. Cross sections of the cord will reveal internal structures of both sides of the cord.

E. Specimen Storage

The usual practice in the preparation of biological materials for microscopy is to fix, process, and embed tissues immediately after they are excised. However, in some cases due to unavoidable circumstances, it is not possible to process the specimens immediately after they are killed or excised from the source, so they must be stored for a period of time. The necessity of storage may arise when it poses a possibility that special detection techniques (e.g., for myelin damage) or electron microscopy may be needed after the general histopathological evaluation (H & E or toluidine blue) is performed. Half of the tissue samples can be fixed and stored in 10% buffered formalin (for light microscopy) and the other half of the specimen can be fixed and stored in buffered glutaraldehyde or paraformaldehyde (for possible electron microscopy investigation). Sabatini et al. (1963) pointed out that tissues can be stored in various aldehydes up to several months without any significant adverse effect on the fine structure. Yamamoto and Rosario (1967) also reported that tissue can be stored in buffered formaldehyde (paraformaldehyde) for over one year without undesirable structural changes. Actually, tissues previously well fixed with aldehydes can be transferred for storage in cold buffer, containing sucrose, up to several months.

Washing the stored tissues with a buffer solution (for electron microscopy) or with water (for light microscopy) prior to tissue processing is a critical factor in obtaining good sectioning.

F. General Histopathological Evaluation

The basic objective of this part of the examination is to detect any morphological lesions in cells and tissues within the resolution power of the light microscope by means of a general staining method.

1. Tissue Sampling.

The frontal cuttings of the brain and the cross sections of the cord (C_3-C_6, L_1-L_4) prepared during gross examination can be subjected to embedding and light microscopic examination. Those tissues from the peripheral nervous system which are known to be vulnerable to neurotoxicants are also included in the screening. All these tissues comprise a fairly good general representation of both the central and peripheral nervous systems. This general tissue survey is required only for the investigation of potential neurotoxicants whose toxic effects are still unknown. For known neurotoxicants, only selected pertinent tissues are needed for the study.

2. Tissue Processing.

All the basic procedures for tissue processing (fixation, dehydration, clearing, embedding, and sectioning) are fairly standard. No special justifications are needed.

It is recommended that multiple tissues can be embedded in one single paraffin block for sectioning. This practice is found to be efficient not only in labor effort (sectioning and staining), but also in storage space (tissue blocks and slides) as well as in material costs. It also saves the pathologist's time in examining multiple tissues in one single microscopic slide.

3. Paraffin/Hematoxylin-Eosin (H & E) or Epoxy/Toluidine Blue Technique as General Screening Method.

H & E is recognized as one of the most useful techniques for general tissue screening in pathology. With paraffin embedding, not only larger tissue samples (e.g., entire cross sections of the rodent brain) can be embedded and sectioned, multiple tissues (e.g., brain, cord, ganglia, etc.) can also be embedded together in one single block for sectioning and staining. Paraffin embedded sections can also be subjected to special staining techniques when that becomes necessary. It is therefore a good, reliable, and cost-efficient technique.

Epoxy embedded sections stained with toluidine blue can be substituted for the H & E technique as a rapid, routine screening method under certain conditions (Spencer and Schaumburg 1980). This procedure is actually a "thick" section preparation for electron microscopy. Sections can be screened with light microscope and the tissue block further trimmed for sectioning. However, this technique can be applied only to a relatively small area of tissue sample and is therefore not very useful unless the precise site of the toxic action is known. Thus, this approach may not be appropriate for the study of unknown neurotoxicants. Tissues subjected to epoxy embedding also require osmium tetroxide fixation and staining and cannot be subjected to further staining with other special neurohistochemical methods. Because it requires epoxy embedding and osmium tetroxide staining, it is also more costly than the basic H & E method. However, very thin (1.0 micron) sections can be generated from the epoxy embedded tissues which provide much higher resolution power than the paraffin sections. Thus the epoxy/toluidine blue method will be a more sensitive method than the routine H & E method and can detect histological lesions more readily. Because of the sensitivity of this method, the need of special detection techniques (stains) may be alleviated.

4. Data Collection, Evaluation and Reporting.

The general guideline implemented in this test standard is consistent with those provided in previously published guidelines. It promotes clear and efficient data collection, note-keeping, and morphological data evaluation and statistical analysis.

IV. SPECIFIC DETECTION AND EVALUATION TECHNIQUES

A. Need for Special Techniques

It has recently been stated by Dr. J. B. Cavanagh, the eminent British neuropathologist that "special techniques are needed to show the cellular details of damage to brain and nerve. An 'H & E', while adequate for most general pathology, is insufficient to supply the answers to neuropathological problems. The special techniques and the special knowledge are reasons for the separation of neuropathology from its parent field." (Spencer and Schaumburg 1980).

Indeed, while H & E is an excellent general stain, it is a very non-specific dye, staining nuclei blue and all other tissue components pink. Therefore, unless the lesions involved are of obvious or extensive nature, paraffin/H & E method may fail to detect changes involving the complex structures and all the tissue components of the nervous system. Special techniques are useful to selectively detect and precisely diagnose changes in the various cellular and tissue components in the nervous system; axons, dendrites, Nissl patterns of the neurons, myelin sheath, etc. However, if epoxy/toluidine blue method is employed, the high sensitivity of this method will enable rapid detection of the lesions without further special techniques.

Contrary to many beliefs, most of the special neurohistochemical techniques are actually quite inexpensive and easy to perform. Most can be done with paraffin sections. Thus additional sections can simply be obtained from the tissue block when sections for H & E staining are being made. The "additional" time for an experienced technician to cut one section (for H & E staining) versus a ribbon of several sections (for special stains) is probably 2-3 minutes per block. The chemicals required for these special stains are readily available and are not particularly high priced. Furthermore, multiple slides as well as multiple tissues on one slide can be stained together at the same time in one staining rack; thus the additional labor time is also not excessive.

B. General Objective of the Special Techniques

The objective of this segment of the study is to use well established and reliable neurohistochemical methods to identify and better define specific structural damages of the nervous tissues (neuronal body, axon, myelin sheath, and peripheral nerve fiber), which may go undetected or may be difficult to determine by H & E staining method.

Other tissue elements such as dendrites, glial cells, endothelial cells, neuromuscular junction, and skeletal muscles may change either as a direct toxic impact or as secondary degeneration to the toxic substance. These elements can be examined with general screening methods (H & E or epoxy/toluidine blue). Special techniques for these elements are usually too difficult and costly for basic laboratories and will not be included in this guideline.

C. Methods for Specific Evaluation of Neural Changes

Special techniques are fairly "routine" in many well established neuropathology laboratories. They have been used and proven to be very helpful in detecting many changes involving the nervous system, such as: early destruction of neuronal Nissl substances (gallocyanin stain) as seen in methylmercury poisoning (Chang and Hartmann 1972), axonal changes (Bodian stain) as observed in IDPN intoxication (Chou and Hartmann 1964), segmental demyelination (nerve fiber teasing technique) as seen in lead poisoning (Fullerton, 1966) and peripheral nerve fiber changes as seen in acetyl ethyl tetramethyl tetralin intoxication (Spencer et al. 1980), neurofibrillary changes (Bielchowsky stain) as seen in aluminum intoxication (Klatzo et al. 1965), and primary or secondary destruction of myelin sheaths (Kluver's Luxol Fast Blue Stain) as seen in human subacute myeloopticoneuropathy (Shiraki 1977) and in clioquinol intoxication (Ikuta et al. 1977). Many of these lesions may go undetected or unidentified (particularly in mild pathological situations) by H & E method. Therefore, special techniques, when used properly, play an important role in neuropathology evaluation.

Positive control slides are needed for each of the special stains to guard against false-positive or false-negative results. These slides must be done together with the test tissues in each staining procedure to assure the validity of the technique.

D. When to Perform

As stated earlier, because of the high resolution and sensitivity of the epoxy/toluidine blue method, special stains may not be needed for animal tissues screened by that technique. However, special techniques have meritorious value for paraffin embedded sections.

Most neurotoxicants are fairly site and action specific, and may be characterized according to their primary or major toxic consequences: neuronopathy (e.g. methylmercury on cerebellar granule cells and dorsal root ganglia; trimethyltin on hippocampal neurons), axonopathy (e.g. acrylamide on distal axons; IDPN on proximal axons), and myelinopathy (e.g. lead on Schwann myelin sheaths; triethyltin on central myelin). Therefore, probably only one or two special techniques will be employed for a given study on known neurotoxicants, e.g., for peripheral nerve degeneration (e.g., acrylamide poisoning), nerve fiber teasing technique will suffice. In the situation of proximal axonal swelling as seen in IDPN intoxication, a combined staining of Bodian/LFB will be enough to detect small axonal swellings (low dose condition) which may go undetected by H & E method and to demonstrate the "swollen, round structures" are myelinated axons and not swollen dendrites (dendritic torpedoes) or tangential cuts of neuronal bodies. Secondary or other minor lesions produced by a neurotoxic compound should be noted with H & E stained sections and do not require detailed special technique screening.

For testing of a potential neurotoxicant whose toxic action is not known, a very high dose or acute exposure group may be included in hope of inducing a more dramatic lesion which can be easily detected by H & E staining method. Once the general nature and site of lesion is known, epoxy/toluidine blue method can be performed on these isolated tissues, or appropriate special techniques for paraffin preparations can then be selected accordingly. If there is absolutely no clue that can be obtained either from the H & E stained sections at any dose level, from the general chemical structure and characterization of the test compound, or from the neurological symptomology as to the probable general nature of the toxic impact, the entire battery of special techniques will have to be used. Situations as such are very rare indeed, because if a chemical compound is neurotoxic, some form of morphological lesion will usually be produced in high dose situations. The implementation of this requirement of using the entire battery of special techniques for paraffin preparations in the test standard is necessary and serves as a safeguard for such rare, but possible, situations.

E. Test Scheme

Two test schemes can be suggested: One is used for known neurotoxicants, the other for suspected neurotoxicants. The H & E stain may be used for both test schemes to the limit of its capacity to detect a lesion. Then the true "no effect level" maybe confirmed by examining the H & E "no effect" tissue with more sensitive, special stains. Alternatively, the epoxy/toluidine blue technique may be used for known neurotoxicants or after H & E has been used to identify the general nature and site of the lesion.

A summary of these test schemes is presented in the flow charts (see Figures 1 and 2).

FIGURE 1 GENERAL TEST SCHEME FOR "KNOWN" NEUROTOXICANT

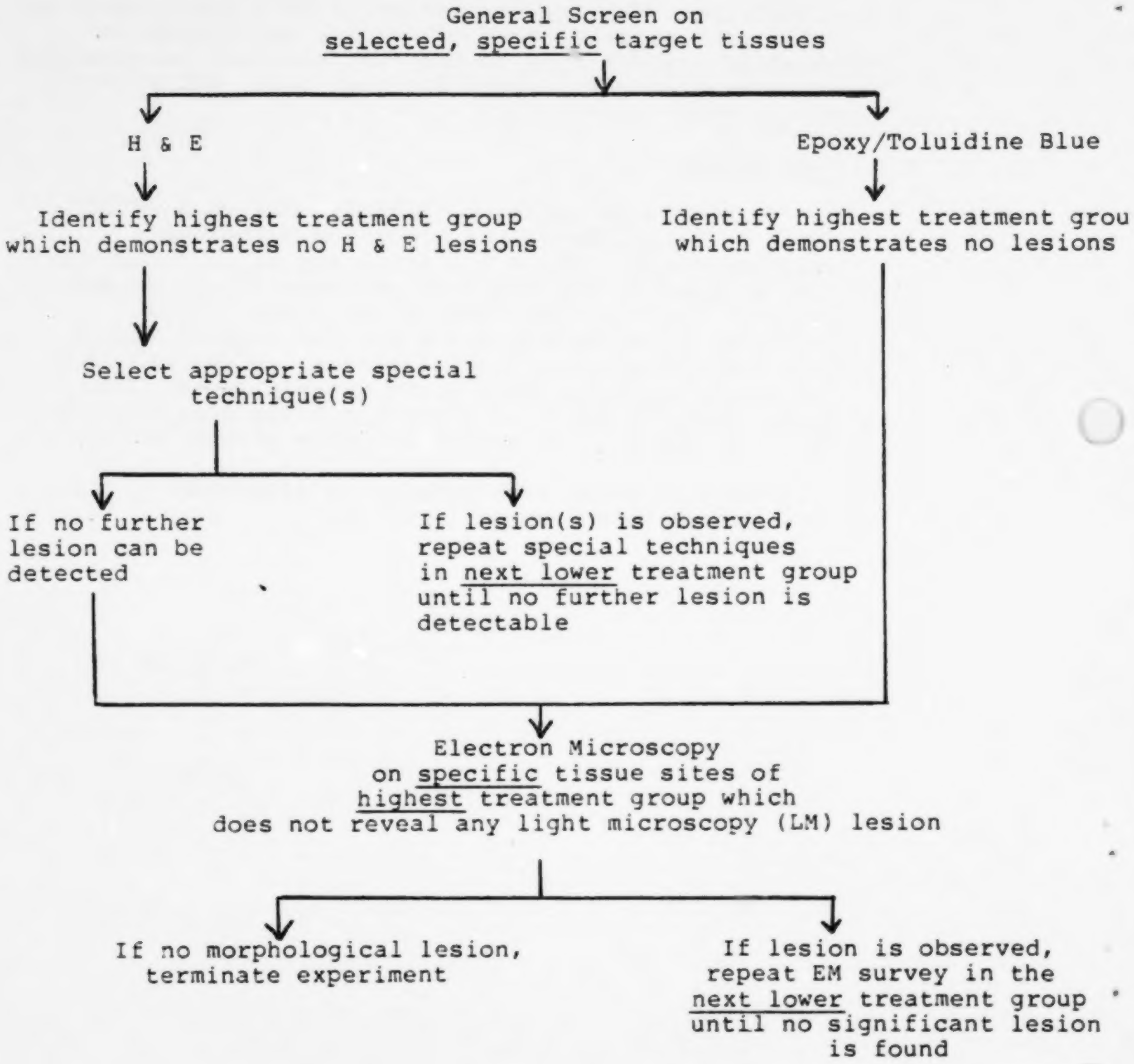
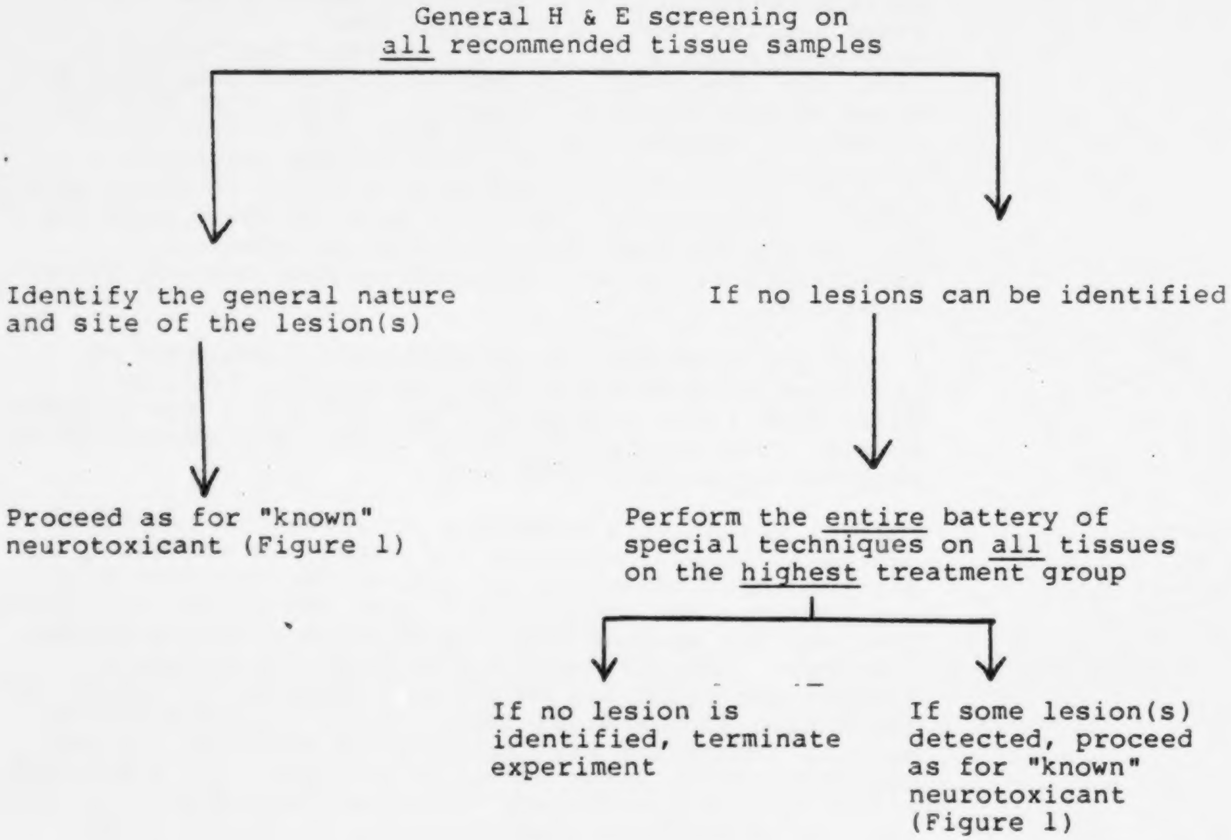

General Screen on selected, specific target tissues
H & E
Epoxy/Toluidine Blue
Identify highest treatment group which demonstrates no H & E lesions
Identify highest treatment grou which demonstrates no lesions
Select appropriate special technique(s)
If no further lesion can be detected
If lesion(s) is observed, repeat special techniques in next lower treatment group until no further lesion is detectable
Electron Microscopy on specific tissue sites of highest treatment group which does not reveal any light microscopy (LM) lesion
If no morphological lesion, terminate experiment
If lesion is observed, repeat EM survey in the next lower treatment group until no significant lesion is found

FIGURE 2 GENERAL TEST SCHEME FOR "SUSPECTED" NEUROTOXICANT



General H & E screening on all recommended tissue samples

Identify the general nature and site of the lesion(s)

Proceed as for "known" neurotoxicant (Figure 1)

If no lesions can be identified

Perform the entire battery of special techniques on all tissues on the highest treatment group

If no lesion is identified, terminate experiment

If some lesion(s) detected, proceed as for "known" neurotoxicant (Figure 1)

F. Economy of The Special Evaluation Methods

It is the intent of this guideline to obtain maximal information with minimal but detailed screening. Data generated should be useful not just to demonstrate that some form of morphological lesion exists, but also be able to accurately define the nature of the lesion, to provide morphological support and correlation with other studies (e.g., behavioral and neurophysiological), and to elucidate the "safety" level (no observable lesion) of the test substance in terms of morphological judgment. Thus, with all the efforts in economizing (both cost and effort) the investigation in mind, the test scheme suggested represents a carefully designed approach to cover all possible situations. Granted, some of the procedures will rarely be used, but they are implemented to safeguard a neurotoxic compound passing the screening undetected.

A chemical compound can be confidently declared as producing no observable light microscopy lesions at a given dose level if and only if both H & E and selected special technique(s) failed to reveal any morphological abnormality at that dose level.

Special stains will certainly increase the number of slides to be cut, stained, and read. As stated earlier, preparation of the additional sections will not increase the technician's time significantly. Time involved for special staining differs with the stains involved. However, multiple slides with multiple tissues per slide can be stained together to reduce the labor time and chemical cost. Since only one or two special techniques will probably be required for one treatment group under normal situations, the additional slides generated will not be exceedingly great. A well trained pathologist should be able to read a slide under 2-3 minute's time (particularly for special stains which define the lesion very sharply). The additional time for slide reading is therefore also within reason. Compared to other screening methods (e.g., behavioral studies), pathology involves much less time and cost.

V. ELECTRON MICROSCOPY

A. Objective

The basic objective of this portion of the test standard is to establish a standard approach in using the most modern and sensitive diagnostic tool available to provide morphological information on the existence or non-existence of ill-effects of a compound at given dose level(s) on specific tissues.

B. Limitations and Advantages

Because of the smallness of the tissue size which can be examined, it is impractical to use EM as a general screening tool. But because of its high resolution power; it can be used to screen and detect subtle morphological lesions (subcellular changes) even at very low dosage levels on specific areas of selected tissue samples which are known to have light microscopic lesions at much higher dose levels. In other words, it can be used to determine whether a "no observable lesion" situation by light microscopic criteria is truly free of any morphological toxic change or it is merely due to the "insensitivity" of the light microscopic resolution. The conclusion of no morphological (pathological) change can be confidently drawn if and only if no detectible structural change can be found even with the most sensitive instrument (EM) available.

The functional significance of subtle morphological changes (e.g., mitochondrial swelling or synaptic abnormality) cannot be determined by morphological techniques. Such observation only provides morphological information on structural changes in the organelles, cells or tissues in the nervous system under certain toxic conditions. Although such changes may or may not exert an immediate or apparent functional deficit to the animal (as stated earlier, the nervous system is an extremely plastic organ which can compensate for many functional deficits despite significant structural damage), such information is extremely important in revealing the early toxic impact of the chemical in question. Physiological and/or behavioral studies would help to elucidate the functional aspect of the organism following toxic exposures.

The only possible way to increase the "sensitivity" of electron microscopy is probably by means of morphometric analysis (quantitative morphology) where extremely subtle changes in the number, size, or distribution of cells or organelles can be estimated as a consequence of toxic influence on the biological system. Because this technique may be too time consuming and costly to average investigators, it is not included in this guideline.

C. When to Perform

Because of the small tissue size that can be examined by electron microscopy, EM should not be used as a "general" screening tool, but rather, should be limited only to screen specific areas on selected tissue samples where lesions are known by light microscopy to occur at higher dose levels. EM study is not needed in any test group(s) demonstrating LM observable pathology. Since the precise tissue site(s) of the lesion(s) will be very well defined by light microscopy at higher dosage levels, usually only one or two selected tissue sites are needed for EM survey at low dose situations. Thus, the actual workload for EM work is really not very much for any given test.

D. Test Scheme

For investigation of substances with known toxic impact, select tissue samples from the highest dose group where no light microscopic lesion is detected should be first subjected to EM study. If EM lesions are observed, tissues from the next lower dose group should also be examined. However, if no EM lesion is found, no further EM study is needed.

A similar approach may be exercised for the study of potential neurotoxicants. EM is performed on specific and selected tissues if and only if LM lesions can be demonstrated at higher dose levels. No EM study is necessary if no detectible LM lesion, both by H & E and by special histochemical stains, is observed at all dose levels studied.

A general test scheme is provided as flow charts (see Figure 1 and 2).

E. Elements to be Examined

The elements recommended for examination are all basic cellular/tissue components of the nervous system.

Since specific structural differences of some elements/organelles may be species related, control animals must be used at all times as reference tissues.

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HG-Neuro-Peri Nerve
October, 1983

PERIPHERAL NERVE FUNCTION

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

The techniques in this guideline are designed to develop data on neurophysiological changes in the nervous system for chemical substances and mixtures subject to such testing under the Toxic Substances Control Act. The data will characterize the neurophysiological changes produced by substances known to be peripheral neurotoxicants and determine dose-effect. The EPA will use these data to assess the risk of neurotoxic effects these chemical may present to human health.

II. DEFINITIONS

A. Neurotoxicity or a neurotoxic effect is an adverse change in the structure or function of the nervous system following exposure to a chemical agent.

B. Conduction velocity is the speed at which the compound nerve action potential traverses a nerve.

C. Amplitude is the voltage excursion recorded during the process of recording the compound nerve action potential. It is an indirect measure of the number of axons firing.

III. PRINCIPLE OF THE TEST METHOD

The test substance is administered to several groups of experimental animals, one dose being used per group. The peripheral nerve conduction velocity and amplitude are assessed using electrophysiological techniques. A dose-effect function is determined.

IV. TEST PROCEDURE

A. Animal Selection

1. Species and Strain

Testing should be performed on a laboratory rodent unless such factors as the comparative metabolism of the chemical or species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies, dictate otherwise. All animals should have been laboratory-reared to ensure consistency of diet and environmental conditions across groups and should be of the same strain and from the same supplier. If this is not possible, groups shall be balanced to ensure that differences are not systematically related to treatment.

2. Age and Weight

Young adult animals (at least 60 days for rats) must be used. Age (± 15 days for rats) must not vary across groups. Weights should be within ± 10% of the mean.

3. Sex

Either (or both) sex(es) may be used. Sex must not vary across groups.

B. Number of Animals

Sufficient numbers of animals shall be used to detect a 10% change from normal conduction velocity at the 5% level with 90% power. Generally, 20 animals/group will satisfy this requirement.

C. Control Groups

1. A concurrent control group is required. This group must be an untreated group, or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

2. A satellite group may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

D. Dose Levels and Dose Selection

At least 3 doses, equally spaced on a log scale (e.g., 1/2 log units) over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve. The highest dose shall produce 1) clear effects on nerve conduction velocity and/or amplitude or 2) life-threatening toxicity. The data from the lower doses must show either 1) graded dose-dependent effects at 2 dose levels or 2) no effects at 2 dose levels, respectively.

E. Duration of Testing

The exposure duration will be specified in the test rule. This will generally be 90 days exposure.

F. Route of Administration

The test substance shall be administered by a route specified in the test rule. This will usually be the route most closely approximating the route of human exposure. The exposure protocol shall conform to that outlined in the appropriate acute or subchronic toxicity guideline.

G. Combined Protocol

The tests described herein may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination.

H. Study Conduct

1. Choice of Nerve(s)

The nerve conduction velocity test must separately assess the properties of both sensory and motor nerve axons. Either a hind limb (e.g., tibial) or tail (e.g., ventral caudal) nerve must be chosen. Response amplitude may be measured in a mixed nerve.

2. Preparation

a. *In vivo* testing of anesthetized animals is required. A barbiturate anesthetic is appropriate. Care should be taken to ensure that all animals are administered an equivalent dosage and that the dosage is not excessive. If dissection is used, extreme caution must be observed to avoid damage to either the nerve or the immediate vascular supply.

b. Both core and nerve temperature must be monitored and kept constant (±0.5°C) during the study. Monitoring of skin temperature is adequate if it can be demonstrated that the skin temperature reflects the nerve temperature in the preparation under use. Skin temperature should be monitored with a needle thermistor at a constant site, the midpoint of the nerve segment to be tested.

c. Electrodes.

(1) Choice of Electrodes.

Electrodes stimulation and recording may be made of any conventional electrode material, such as stainless steel, although electrodes for non-polarizing materials are preferable. If surface electrodes are used, care must be taken to ensure that good electrical contact is achieved between the electrode and the tissue surface. Following each application, any electrode must be thoroughly cleaned.

(2) Electrode Placement.

Electrode placement must be constant with respect to anatomical landmarks across animals (e.g. a fixed number of mm from the base of the tail). Distances between electrodes used to calculate conduction velocity must be measurable to ± 0.5mm. The recording electrodes should be as far from the stimulating electrodes as possible. A 40 mm separation is adequate in the caudal tail nerve of the rat.

(3) Recording Conditions.

The animal should be grounded at about the midpoint between the nearest stimulating and recording electrodes. The recording conditions must be such that the stimulus artifact has returned to baseline before any neural response is recorded which is used in the analysis, under condition of maximal band width of the preamplifier.

d. The electrical stimulator must be isolated from ground. Biphasic or balanced pair stimuli to reduce polarization effects are acceptable. A constant current stimulator is preferred (and required for polarizable electrodes) and should operate from about 10 uA to about 10 mA. If a constant voltage stimulator is used, it should operate to 250V. All equipment shall be calibrated with respect to time, voltage, and temperature.

e. The recording environment should be enclosed in a Faraday cage unless electromagnetic field pick-up can be shown to be more than 1.5 times the amplifer baseline noise, under recording conditions. The recording output should be amplified sufficiently to render the compound action potential easily measureable with an oscilloscope. The amplifier should pass signals between 2.0 Hz and 4 kHz without more than a 3dB decrement. The preamplifer must be capacitatively coupled or, if direct coupled to the first stages, must be able to tolerate any DC potentials which the electrode-preparation interface produces, and to operate without significant current leakage through the recording electrodes.

f. A hard copy must be available for all waveforms or averaged waveforms from which measurements are derived, and for all control recording required by this standard. Hard copies must include a time and voltage calibration signal.

3. Procedure

a. General

Stimulation should occur at inter-stimulus interval significantly below the relative refractory period for the nerve under study. Stimulus intensity should be increased gradually until the response amplitude no longer increases. At this point the "maximal" stimulus current is determined. An intensity 25-50% (a fixed value in a given study) above the maximal intensity so determined should be used for determining response peak latency and response amplitude. Response peak latency may be read off the oscilloscope following single sweeps or determined by an average of a fired number of responses. The baseline-to-peak height technique (Daube, 1980) is acceptable for determination of the nerve compound action potential amplitude, but in this case, at least 16 responses must be averaged.

b. Motor Nerve

Motor conduction velocity may be measured from a mixed nerve by recording the muscle action potential which follows the compound action potential of the nerve. The stimulus intensity is adjusted so that the amplitude of the muscle action potential is supramaximal. Measurement of the latency from stimulation to the onset of the compound muscle action potential gives a measure of the conduction time of the motor nerve fibers. To calculate the conduction velocity, the nerve must be stimulated sequentially in two places each with the same cathode-anode distance, and with the cathode located toward the recording electrode. The cathode to cathode distance between the two sets of stimulating electrodes is divided by the difference between the two latencies of muscle action potential in order to obtain conduction velocity. Placement of electrodes shall be described-site of nerve stimulation may differ from point of entry through skin.

c. Sensory Nerve

The somatosensory evoked potential may be used to determine the sensory nerve conduction velocity in a mixed nerve. The cathode is placed proximally at the two stimulation locations with the same cathode-anode distances. The recording electrodes are placed on the skull. The conduction velocity is calculated by dividing the distance between the two stimulating cathodes by the difference between the two latencies of the largest priminary peak of the somatosensory evoked potential. Between 64 and 128 responses should be averaged. The stimulation frequency should be about 0.5 Hz. Stimulus intensity should be the same as that used for determining the motor conduction velocity. Should the peak of the somatosensory response be so broad that it cannot be replicated with an accuracy of less than 5% of the latency difference observed, then a point on the rising phase of the potential should be chosen, e.g. at a voltage 50% of the peak voltage. Alternatively, the sensory nerve conduction velocity can be obtained from a purely sensory nerve or from stimulation of the dorsal rootlets of a mixed nerve, using two recording electrode pairs.

V. DATA COLLECTION, REPORTING AND EVALUATION

In addition to information meeting the requirements stated in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40 Code of Regulations], the following specific information should be reported:

A. Description of Test System and Test Methods

1. Positive control data from the laboratory performing the test which demonstrate the sensitivity of the procedure being used.
2. Hard copies of waveforms from which measurements were made as well as control recordings.
3. Voltage and time calibration referable to the standards of the Bureau of Standards or to other standards of accuracy sufficient for the measurements used.
4. Data demonstrating that nerve temperature was maintained constant throughout the recording period.

B. Results

The following information must be arranged by test group (dose level):

1. In tabular form, data must be provide showing for each animal:
   a. Its identification number;
   b. Body weight, nerve conduction velocity, and amplitude.
2. Group summary data should also reported.

C. Evaluation of Data

An evaluation of the test results (including their statistical analysis) must be made and supplied. This submission must include dose-effect curves for conduction velocity and amplitude and a description of statistical methods. Deviation from conventional parametric techniques must be justified.

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HS-Neuro-Peri Nerve
October 1983

PERIPHERAL NERVE FUNCTION

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
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TABLE OF CONTENTS

Pages

I. INTRODUCTION: NEED FOR STUDIES OF PERIPHERAL NERVE FUNCTION 1

II. RATIONALES FOR STUDY DESIGN 7
- A. Choice of Subjects 7
- B. Choice of Nerve(s) for Test 7
- C. Number of Animals 8
- D. Preparation 8
  - 1. General 8
  - 2. Anesthesia 9
  - 3. Temperature 10
  - 4. Electrodes 10

III. METHODS OF STUDY CONDUCT 14
- A. General Procedure for Determining Conduction Velocity and Amplitude 14
- B. Motor Nerve 16
- C. Sensory Nerve 16

REFERENCES 19

HS-Neuro-Peri Nerve
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I. INTRODUCTION: NEED FOR STUDIES OF PERIPHERAL NERVE FUNCTION

The vertebrate nervous system has both central and peripheral components. The peripheral nervous system is composed of those axons, dendrites, cell bodies and accessory organs not present in the brain or spinal cord. Because of their immediate accessibility, considerable information about the characteristics of peripheral nerves is available, including their anatomy, physiology and response to toxic insult. Many texts are available which describe the general properties of the peripheral nervous system (e.g. Waxman 1978). A few general points are important for orientation purposes and these will be described briefly below.

Peripheral nerves consist of heterogeneous bundles of axons from nerve cells. Most peripheral nerves contain both sensory and motor axons of a variety of diameters from nerve cells in different areas of the body. Information transmission along nerves is by way of action potentials in the axons. In the normal (physiological) condition, axons conduct action potentials in one direction (orthodromically) which is towards the central nervous system for sensory axons, and away from the cell body for motor axons. The larger diameter axons conduct action potentials more rapidly (i.e. have faster conduction velocities) than the small ones. All but the smallest axons have a myelin sheath, which is a fatty external insulation interrupted at regular intervals by nodes of Ranvier. Action potential conduction in myelinated nerves is saltatory, appearing to jump from one node of Ranvier to the next. Saltatory conduction is more rapid than conduction in unmyelinated nerves. Since conduction velocity is related to axon diameter (e.g. Cullhein and Ulfhake 1979), depending in some preparations upon a general geometric factor shown by Mirolli and Talbott (1972) to be equal to $\sqrt{A/P}$, where A is the cross-sectional area and P is the perimeter, and since axon diameter of any particular nerve cell tends to decrease with distance from the cell body of origin (largely due to branching) (Quillian 1956), conduction velocity is not constant along the entire length of the axon.

The ability of an axon to conduct an action potential is contingent upon the integrity of its membrane and the maintenance of ionic concentration differences between the inside and outside of the axon (i.e. across the membrane). Maintenance of the ionic concentration differences depends in part upon the integrity of energy metabolism in the membrane (Hodgkin and Keynes 1955). The greater speed of myelinated nerve conduction is obviously contingent upon the integrity of the myelin sheath and the node of Ranvier.

A number of substances have been demonstrated to produce peripheral neuropathy. Among these substances are organic solvents such as n-hexane (e.g., Scelsi et al. 1980), carbon disulfide (Vigliani 1954), methyl-n-butyl ketone (e.g. Spencer et al. 1975) and acrylamide (LeQuesne 1980). A delayed form of peripheral neuropathy is produced by some organophosphate esters (Barnes and Denz 1953). Peripheral neuropathy has also been reported following lithium intoxication (Uchigata et al. 1981), alcoholism (e.g. Ballantyne et al. 1980), diabetes (e.g. Sharma and Thomas 1974), dapsone exposure (Koller et al. 1977), disulfiram (Moddel et al. 1978), and nitrous oxide exposure (Layzer et al. 1978).

Not all agents which produce peripheral neuropathies do so by the same mechanism. Recent efforts to classify neurotoxic disease according to the cellular target site (Spencer and Schaumburg 1980) have identified three basic types of toxicants: those which affect the cell body (neuronopathy); those which affect the peripheral processes of the cell (axonopathy) and those which affect the myelin sheath covering the axons of some cells (myelinopathy). Most of the toxicants producing peripheral neuropathy have the axon as the primary target, although some produce axonal damage which is secondary to neuronopathy (e.g. doxorubicin, Cho et al. 1980). Substances producing primary toxicity in axons may selectively affect either the distal axon (e.g. hexacarbons, carbon disulfide, acrylamide and TOCP, Spencer and Schaumburg 1980) or the proximal axon (e.g. β,β'-iminodipropionitrile; Spencer and Schaumburg 1980). Substances which preferentially attack myelin also produce peripheral neuropathy (e.g. hexachlorophene; Towfighi et al. 1973).

Unfortunately it is not yet possible to predict the type of toxic effect from the chemical structure of the compound. For example, two structurally similar alkyltin compounds produce strikingly different toxic effects. Triethyltin produces a myelinopathy (Torack et al. 1970), while trimethyltin produces a neuronopathy (Brown et al. 1979). Tests for detection of peripheral nerve dysfunction must therefore be sufficiently sensitive and generalized to detect these different types of effects. A growing number of studies have used the well developed science of electrodiagnosis to detect neuropathy. The techniques available in this science have been used in neurological clinics for some time, but have been applied less frequently to detection of neurotoxicity in experimental animals.

The techniques of electrodiagnosis allow the direct measurement of activity in nerves. Production of changes in electrical potential during activity is a general property of axons. Details of the properties of these axons are available in any physiology text (e.g. Mountcastle 1968). Briefly, it can be stated that an axon responds to a stimulus in an all-or-none way. When stimulated, either via postsynaptic potentials from its cell body, generator potentials at its terminals, or an electrical stimulus applied in the vicinity of the axon itself, an axon either transmits or does not transmit an action potential i.e. responds in an all-or-none-fashion. Barring toxic or extrinsic interference or stimulation in the relative refractory period, once an action potential is triggered in a particular axon, its conduction properties are exactly like all other action potentials which have been triggered in that axon: it has the same speed and amplitude. Since nerves are made up of bundles of axons with different thresholds for production of an action potential, and with different conduction velocities, an electrically stimulated nerve does not produce an all-or-nothing response in the same sense that an axon does. The action potential produced by stimulation of a nerve is called the compound action potential, because it contains the compounded activity of the axons within it. Following any particular stimulus, none, some, or all of the axons in the nerve might produce action potentials. Whether a particular axon produces an action potential depends upon its thresold, its proximity to the stimulating electrode, and whether the axon is in a refractory period from any previous stimulation. In a given experiment, when stimulation is at a low rate (e.g. less than 25 hz) and electrode position is unchanged, the only variable which should alter the presence or absence of an action potential from a particular axon is stimulus intensity. The compound action potential recorded from a nerve represents the summated activity of all the axons stimulated by the stimulus. As stimulus intensity increases, more axons will reach their thresholds for action potentials, and thus the amplitude of the response may be expected to increase. When the stimulus is sufficiently intense to have activated all axons in the nerve, further increases in intensity will no longer increase the amplitude of the response.

The latency from the time of supramaximal stimulation to the time at which a compound action potential is recorded from the nerve depends upon the distance between the stimulating and recording electrodes (assuming both are close to the nerve) and the conduction velocity of the stimulated axons in the nerve. Rapidly conducting axons have lower thresholds than slowly conducting axons and, therefore, at low stimulus intensities only the more rapidly conducting axons will be recorded. Whether a particular axon's action potential contributes to the compound action potential is in part contingent upon whether the recording electrode is close enough to detect it. An action potential may occur in part of the nerve which is relatively distant from the recording electrodes. In that case, its contribution to the amplitude of the compound action potential will be small. If it is a relatively rapidly conducting action potential, its peak will occur before the peak of the compound action potential. Thus the ascending slope of the compound action potential is composed of the ascending slope of action potentials which peak before, shortly after, and at the same time as the peak of the compound action potential. The peak of the compound action potential represents the time at which the most fibers are active at the recording site. As such, measurement of the latency from stimulus to peak of the compound action potential reflects conduction velocity in the population of axons best represented in the nerve (Hursh 1939). To assess the most rapidly conducting axons, latencies must be calculated to the onset of the compound action potential.

The duration of the compound action potential also depends upon the stimulus intensity. As higher stimulus intensities are used, fibers with a wider range of conduction velocity are stimulated and therefore at the recording electrodes some responses arrive relatively early (large low threshold fibers relatively distant from the stimulus), while most arrive relatively late (small higher threshold fibers). The net effect of intensity increases is prolongation of the recorded response.

Any process which alters the distribution of fiber types within a nerve (i.e. selectively destroys large or small fibers) will obviously shift the conduction velocity of the whole nerve response. Equally obvious is that, as fibers are destroyed, the amplitude of the compound nerve action potential will decrease in proportion to the destroyed fibers' normal contribution to the compound action potential. Finally, any process which alters the membrane properties of the axon will change the stimulus requirements for elicitation of a maximal response.

Assessment of the functional integrity of the peripheral nervous system with the techniques of electrodiagnosis (i.e. neurophysiologically), may take many forms. Among the techniques which have been utilized are dissection of single axons (e.g. Mitolo-Chieppa and Carratu 1980), assessment of refractory period (Lowitzsch et al. 1981, Hopf and Eysholdt 978), assessment of the extent to which axons and nerves can follow trains of stimuli which occur at high rates (Lehmann and Tachmann 1974), measurement of peripheral nerve reflexes such as the H reflex and responses such as the F response (Lachman et al. 1980), accomodation indices (Quevedo et al. 1980), and use of collision techniques for selectively blocking activity of some nerve axons to study others (Kimura 1976). Some of these techniques may become useful in assessment of toxic neuropathy in the future. The present standard, however, focuses upon three measures of the functional integrity of the nerve which have demonstrated utility in both the neurological clinic and in detection of toxic neuropathy: conduction velocity, response amplitude, and chronaxy.

Conduction velocity is the speed at which action potential are conducted along axons. As indicated earlier, conduction velocity of an axon is dependent upon the diameter and myelin sheath of the axon, and conduction velocity of a nerve is dependent upon which axons in the nerve are stimulated. Conduction velocity is usually measured in such a way that the activity of the fastest conducting axons is assessed. Changes in conduction velocity which occur following exposure to toxic agents producing an axonopathy are reliable, but usually not large, often ranging from 10% to 30% of control values (Gilliatt 1973). On the other hand, demyelination produces large decrements (>50%) in conduction velocity (McDonald 1963). It is therefore reasonable to assume that if large decrements in conduction velocity have occured, either the nerve contains a large number of demyelinated axons, or the population of large diameter axons in the nerve has been greatly reduced.

As indicated earlier, the amplitude of the recorded compound action potential is related to the number of axons in the nerve which have activated. The only way to assess amplitude is to ensure that all of the axons capable of producing action potentials are in fact responding. Otherwise, differences in amplitude between groups may simply reflect differing thresholds of stimulation for the different populations of axons. One may ensure that all excitable axons in the nerve are stimulated when further increases in stimulating current fail to produce increases in response amplitude. It is generally recognized that decreases in amplitude of the compound nerve action potential occur in both axonopathy and myelinopathy. The two conditions are most easily differentiated by observing the concommitantly greater reduction in conduction velocity which occurs with myelinopathy (Daube 1980).

Chronaxy determination in muscle is a routine procedure in clinical electromyography and is useful for detecting denervation (Rogoff 1980). Determination of chronaxy in peripheral nerves is less common, but allows assessment of excitability of the nerve. A number of natural (e.g. tetrodotoxin), and man made (e.g. DDT), toxicants affect membrane properties of neurons.

Alterations in chronaxy may be expected to aid in detection of these effects, however the precise nature of the effect will not be discernible from an altered chronaxy. Detailed characterization of toxic effects would have to be carried out by other techniques, such as voltage clamping of the membrane (Narahashi 1980).

Aside from their clinical utility, the techniques of electrodiagnosis have demonstrated sensitivity to neurotoxicity. Deficits in conduction properties of rat peripheral nerves have been demonstrated following exposure to a variety of substances, including acrylamide (Boyes 1980), n-hexane (Robert 1981), carbon monoxide (Petajan et al. 1976), hexachlorophene (Maxwell and Le Quesne 1979), and carbon disulfide (Seppalainen and Haltia 1980).

II. RATIONALES FOR STUDY DESIGN

A. Choice of Subjects

Few *a priori* reasons can be developed for requiring the use of a particular species for these tests. Rats and mice have particular advantages since they are relatively cheap and available. Since a moderately large sample size (e.g., 20 subjects/group) is required to detect changes this may make the use of rodents generally preferable. Organophosphate compounds (e.g., TOCP) known to produce delayed axonopathies in some species do not produce delayed neurotoxicity in rodents. The effects of organophosphate compounds may only be detected using hens or other species of known sensitivity to that type of toxicant. Due to the ease with which long nerves are studied compared to short nerves, many investigators may choose large animals such as cats. Use of the cat may make *in vitro* recordings from the sural nerve (pure sensory) more feasible.

Regardless of the species chosen, it is important to use animals from the same strain, of the same gender, and of the same age (± 15 d for rats), since these variables have been shown to influence nerve conduction properties (Miyoshi and Goto 1973, Glatt et al. 1979, Hegmann 1975).

B. Choice of Nerve(s) for Test

The nerve conduction test should assess the properties of at least one sensory nerve and one motor nerve. Many peripheral nervous system disturbances related to toxicants are first detected in sensory nerves (e.g. Schuchmann and Braddom 1980). Since long nerves are generally affected earlier than short nerves (e.g., Barnes and Denz 1953) (perhaps owing to their larger diameter), either a hindlimb or tail nerve should be chosen. The caudal tail nerve of the rat is probably the easiest with which to work, and the description of methods which follow is therefore oriented towards the use of this nerve. The methods described are sufficiently general that they may be easily adapted to different nerves in different species.

C. Number of Animals

Details of the experimental design will depend upon whether a particular study is to involve acute or repeated exposures. It is not the purpose of this standard to specify precise exposure parameters or number of groups to be used. However, in any study it will be necessary to include an untreated (vehicle treated) and a positive control group, i.e., a compound and dosage known to affect peripheral nerve function. The number of subjects to be tested in each group must be considered. The methods described so far have specified conditions that would allow differences in conduction velocity on the order of 10% to be detected. In the rat caudal tail nerve, 2 m/sec represents about 10% of the normal conduction velocity (100 day old rats, temperature controlled at 37°C- Miyoshi and Goto 1973, Glatt et al. 1979). Using the rough rule of thumb that means whose standard errors do not overlap are probably significantly different, it is clear that a 10% alteration in conduction velocity will not be detected unless each standard error is less than 5% of the mean. Thus, in order to detect a 4 m/sec difference in conduction velocity, enough subjects should be tested to ensure that the standard error of the mean is less than 5%. In actual practice (e.g. Miyoshi and Goto, 1973), a group size between n = 10 and n = 25 should achieve such a standard error, but the actual group size must depend on the variability achieved in the individual laboratory. A greater standard error (±10%) is acceptable for amplitude measurements, and should be achieved with the same group size as required for conduction velocity measurements. Statistical analysis of the acquired data should be by conventional parametric techniques, presumably analysis of variance.

D. Preparation

1. General

In vivo testing of nerve function is desirable to ensure comparability in chronic, subchronic, and acute studies. Numerous studies have indicated the feasibility of serial testing of the rat caudal nerve (e.g., Glatt et al., 1979, Miyoshi and Goto, 1973, and Rebert, 1981). Although it is possible to adequately perform tests using isolated nerves, many more subjects may be required since no within-subject designs would be possible. Variability is apparently not decreased by using in situ dissected nerve preparations (e.g., compare Rasminsky et al. 1978, with Glatt et al. 1979). In using the in vivo preparation, use of anesthetized animals is recommended. Use of the unanesthetized preparation would be preferable except that the procedure produces discomfort in the animal which can be avoided by anesthesia. Unanesthetized preparations should be used only when it is presumed that the experiment will be compromised by the anesthetic, or it can be demonstrated that the animal is not in distress.

2. Anesthesia

General anesthetics are known to block nerve conduction, but depending upon the anesthetic there is a varying degree of safety between the concentration which produces general anesthesia and the concentration which produces blockade of nerve conduction (Barker 1975). This margin of safety is especially large for the barbiturate anesthetics. In the rat caudal tail nerve, no change in conduction velocity occurs following 20 mg/kg sodium pentobarbital. A 3.5 m/sec decrease in velocity occurs following 40 mg/kg but no further decline is evident at 60 mg/kg (Glatt et al. 1979). Therefore, barbiturate anesthetic is appropriate. Care should be taken to ensure that (1) all animals are administered an equivalent dosage, and (2) the dosage administered is not more than is necessary to maintain a level of general anesthesia. Investigators should be alerted to the possibility that the appropriate dosage may be influenced by the toxic effects of the test substance. For rats and mice, a combination of sodium pentobarbital and chloral hydrate (Chloropent, Fort Dodge) is effective at 3.5 ml/kg body weight (i.p.). A dosage of 50 mg/kg sodium pentobarbital with 0.1 mg/kg atropine pre-treatment is also adequate. Some anesthetics, such as urethane, seem to offer an advantage since their depression of peripheral reflexes is less than barbiturates, and the level of anesthesia is apparently stable for a longer period of time than sodium pentobarbital. Urethane, unfortunately, produces liver damage and therefore renders the session terminal for the subject.

3. Temperature

Temperature is a critical variable which must be monitored and maintained during the experiment. Conduction velocity may drop as much as 2.4 m/sec for every C drop in temperature (Davis et al. 1975, Braddom and Schuchmann 1980). Further, it is well known that some many anesthetics impair thermoregulation (see Goodman and Gilman 1980). An acceptable study must demonstrate that temperature has been adequately controlled. An effective way to do this is to immerse the tail (if the tail nerve is used) or the nerve (if a dissected *in vivo* or *in vitro* preparation is used) in a warm mineral oil (or other non-conducting fluid) bath, maintained at constant temperature. The tail (or nerve) must remain in the solution during testing, and should be in the solution for at least 5 min (Miyoshi and Goto 1973) before testing is begun, to allow warming up to the constant temperature of the bath.

4. Electrodes

Electrodes for stimulation and recording may be made of any conventional electrode material. An effective electrode for recording from the rat tail nerve is the needle electrode commercially available from a number of suppliers. Different electrode materials have different impedances, and this may affect response amplitude. If the electrode type remains constant within an experiment, there will be no variation in response amplitude as a result of changing electrode types. If surface electrodes are used in place of needle electrodes, care must be taken to ensure that good electrical contact is achieved between the electrode and the tissue surface. The tissue should be cleaned and an electrolyte gel applied to the tissue-electrode junction. The use of surface electrodes is more difficult when bath immersion is used for heating, since leakage of the bath fluid into the tissue-electrode junction will significantly reduce conductivity. If surface electrodes are used, the investigator must be able to demonstrate that good electrical contact was maintained. An acceptable method for accomplishing this is measurement of tissue impedance. In addition, the DC potential from the electrodes should be monitored unless the amplifier is known to be able to tolerate electrode offsets of up to 800 mV, e.g. by capacitative coupling (Schmitt and Almasi 1971, Patterson 1978). Following each application, any electrode used must be thoroughly cleaned.

Electrode placement and the accurate measurement of interelectrode distances is critical to the success of the method. A scheme must be devised to ensure that electrodes are placed in the same location from one animal to the next. One successful method is to use a plastic block grooved on the underside to form a trough which matches the contour of the tail, with holes penetrating the groove at precisely measured intervals. The holes, which should be just large enough to accept a needle electrode, may be conveniently spaced at 5 mm distances in a line which tracks the length of the tail at approximately the midpoint between the midline and the lateral surface. Electrodes should also always be inserted to a common depth in the tail. Although conduction velocity can be calculated whenever the distance between the stimulating and recording electrodes is known, it is important to keep this distance constant across animals. As the distance between the recording and stimulating electrodes increases, the compound action potential waveform becomes broader due to between-fiber differences in the conduction velocity of fibers within the nerve (Gasser, 1943). Consequently, failure to keep a constant distance between animals may yield erroneous estimates of both velocity and amplitude of the compound action potential.

In order to achieve an accurate assessment of conduction velocities, it is necessary to place the recording electrodes as far away from the stimulating electrodes as possible. If the stimulating and recording electrodes are too close, then the measuring equipment may not be sensitive enough to resolve small differences in peak latency between groups. An adequate distance between the most distal stimulating electrode nearest the recording electrode (which should be the cathode) and the nearest recording electrode is 40 mm. This distance allows assessment of conduction velocities which may differ by 1 m/sec if the recording device can detect differences of 0.1 msec. However, this determination of conduction velocity assumes that the point of stimulation is accurately known. Under some conditions the stimulating point can be several millimeters from the cathode. A more accurate method utlizes the latency between two recording electrode pairs (with a single stimulation pair), while a somewhat less accurate, but acceptable method, uses the latency difference between a single recording pair and two stimulating pairs. The latter method is required when a response adds an indeterminate delay to the conduction latency, as in the muscle response and the somatosensory evoked response. Since conduction velocity measurements for both motor and sensory fibers must be obtained according to the guideline, the use of two stimulating pairs will generally be used in determining conduction velocity in a mixed nerve by means of a single recording and single stimulating pair will be redundant. Such a recording configuration will be useful in determining action potential amplitude, and peak latency in a mixed nerve, as described in Section III. A. 1. The distance between the anodal and cathodal electrodes should be about 5.0 mm and remain constant throughout the experiment and should be the same for all pairs of stimulating electrodes used on a given nerve. The animal should be grounded at midpoint between stimulating and recording electrodes.

To reduce artifacts, the electrical stimulator used to elicit the compound action potentials should be isolated from ground. The stimuli should be square wave pulses of constant duration; usually between 0.01 and 1.0 msec duration. Pulses which are 0.1 msec are convenient for routine use since they are not long enough to produce a large artifact, but are long enough such that the stimulus intensity does not have to be excessive in order to achieve a supramaximal stimulus. Current flow, not voltage, is the effective simulus for nerve tissue (Ranck 1975) and thus a more precise description of the stimulus is provided when a constant current rather than a constant voltage is used. Furthermore, polarization of electrodes can change current flow from a constant voltage stimulator.

Therefore, a constant current stimulator is desirable. The current range over which a stimulator should operate is from about 10 uA to about 10 mA. If a constant voltage stimulator is used instead of a constant current stimulator, it should operate up to 250 V in order to provide sufficient current to excite abnormal nerves (Daube 1980) and should be provided with a series resistor across which a differential amplifier can measure current.

The recording environment, in addition to including a constant temperature apparatus (described above), may need to be enclosed in a Faraday cage. A grounded Faraday cage will significantly reduce electrical interference from other sources (Wolbarsht 1964).

The recording electrodes should be lead, via a shielded cable, to the input of a differential amplifier. The gain of the amplifer should be sufficient to render the compound action potential easily measurable with an oscilloscope (i.e., produce a peak deflection of 4-5 cm) and at the same time be within the range of appropriate input signal for a signal averager or summator. The amplifier should pass signals between 2.0 Hz and 4kHz without more than a 3dB decrement. A signal averager or computer is necessary to accurately measure the compound action potential amplitude. A large variety of such devices are available. The only constraint is that the device must be capable of sampling at a rate sufficient to detect small alterations in conduction time. A minimum sampling rate is 20 usec/point, or 50 kHz, for conduction distances as short as 40 mm. At this sample rate, a change in conduction velocity from 40 m/sec to 39 m/sec would be detectable. For nerves with faster conduction velocities, this combination of sample rate and segment length would not detect alterations of 1 m/sec.

Hard copies must be available for representative compound action potentials from which measurements are included in a study. These may take the form of oscilloscope tracing photographs, X-Y plotter outputs from a signal averager, or any other suitable device. Hard copies should contain a time and amplitude calibration signal.

III. METHODS OF STUDY CONDUCT

A. General Procedure for Determining Conduction Velocity and Amplitude

In a typical experiment, the procedure should be as follows. All equipment is turned on, checked, and calibrated. The constant temperature bath is heated to the appropriate temperature. The animal (whose group should be blind to the testing technician if possible) is anesthetized, the tail (or nerve) is placed on the recording trough, and the electrodes are inserted. At this point, a check should be made of the adequacy of recording. The tail is then immersed in the mineral oil bath and allowed to warm for at least 5 min (Glatt et al., 1979). When the tail has warmed, the stimulator is turned on and may be used at any convenient rate (e.g., 2-3 hz) which is significantly below the relative refractory period of the nerve. The current is gradually increased until the nerve response is noted on an oscilloscope. The nerve response should be easily detectable before a muscle response becomes apparent. If it is not, the electrode may be repositioned. However, preceding statements regarding electrode position still apply. If a question exists whether a response is the result of nerve or muscle activity, the muscle activity may be fatigued with a high frequency (e.g., 500 hz) stimulus. This procedure is only useful for identification purposes, not data collection. High rates of stimulation would cause stimuli to fall within the relative refractory period of the nerve, and therefore reduce amplitude. As the nerve response becomes apparent, the stimulus intensity is gradually increased until the response amplitude no longer increases. This value of the stimulus is the maximal intensity. The intensity used for studying conduction velocity and response amplitude should be set at some fixed ratio, e.g. 25%-50% above the maximal intensity. For determining conduction velocity, the response latency may either be read off the oscilloscope or, preferably, determined from the averager. An average of 16 responses should be sufficient to produce a reliable estimate of both the latency and amplitude of the compound action potential. In clinical electromyography, the latency for motor conduction velocity studies is usually taken to the onset of the compound muscle action potential wave while the latency for sensory conduction velocity studies is usually taken to the peak of the compound nerve action potential wave (Schuchmann and Braddom

1980). Peak latencies provide an index of the conduction velocity of the average fiber in the bundle, while onset latencies provide an index of the conduction velocity of the fastest fibers in the bundle. Given the occasionally small size of the compound action potential, it may be more expedient to consistently measure the peak latency than the onset latency or to use as the end point voltage at one-half the peak voltage. This measurement is less dependent upon the sensitivity of the recording equipment. It should be noted that measurements derived from the compound nerve action potential in a mixed nerve include both sensory and motor fibers. The conduction velocity is calculated by dividing the conduction distance (from most distal stimulating to most proximal recording electrodes) by the conduction time (peak latency-onset of stimulus artifact).

Two approaches to measuring the amplitude of the compound action potential wave are possible. Either the peak height may be measured from the pre-spike baseline, or the area under the wave may be measured from onset to return to baseline. In either case, it is necessary to make the measurements in a reproducible and accurate manner. Many averagers have a capability of providing a digital output corresponding to a cursor location on the displayed waveform. With these instruments, the peak height is easily determined as the difference in voltage between the peak and the baseline. The peak height gives an estimate of the number of axons with average conduction velocities in the fiber bundle or nerve measured. The area under the wave gives a measure of the total number of functional axons within the nerve. This area may be measured either by a version of the polar planimeter, or by electronic means using either a computer or attachments available on special purpose averagers. Given the added information provided by performing measurements of area, it is recommended as the standard technique. Bipolar recording techniques may produce responses whose amplitude and areas are difficult to measure (i.e. biphasic). In these cases the negative pole of the differential amplifier may be grounded. If chosen for one preparation, this monopolar configuration should be maintained throughout the experiment.

B. Motor Nerve

In most cases, it is appropriate to differentiate between the sensory and motor components of the nerve response. Since the compound action potential of the mixed nerve recorded as described above confounds the orthodromic motor fiber and antidromic sensory fiber responses, it is of minimal value as a measure of conduction velocity although it can provide adequate information on nerve response amplitude, peak latency, and chronaxy. However, extraction of the motor component of the mixed nerve is a simple matter. The muscle response which follows the compound action potential of the nerve results only from activity of the motor fibers in the nerve. Thus, measurement of the latency from stimulation to the onset of the muscle response gives a measure of the conduction time of the motor fibers. This measure is confounded with the synaptic delay between the arrival of the action potentials at the terminals of the axons and the contraction of the muscle. To calculate the conduction velocity, therefore, the nerve must be stimulated in two places. The distance between the two sets of stimulating electrodes (the cathode to cathode distance) is then divided by the difference between the two latencies in order to obtain the conduction velocity. In these studies, the stimulus is adjusted so that the amplitude of the muscle action potential is supramaximal. The amplitude of the muscle action potential is measured by the same means as described for the mixed nerve action potential. Under these conditions, the distance between the proximal and distal stimulating cathodes must be at least 20 mm for sweep sample rates as slow as 50 kHz.

C. Sensory Nerve

Two approaches to recording the conduction velocity of the sensory nerve response are acceptable. In the first, a purely sensory nerve, or the sensory branch of a mixed nerve, is stimulated and the antidromic action potential is recorded a fixed distance from the stimulus as described above. This direct recording method is conceptually the simplest. However, it requires careful dissection and is especially difficult in rodents since the sural nerve (the most appropriate nerve upon which to do the study) is quite short. An acceptable procedure is to stimulate the sural nerve and record from the sciatic nerve trunk in the pelvis. If this procedure is used, great care must be taken in measuring the conduction distance. The sural nerve of cats is a more suitable preparation upon which to perform this study by the direct recording method. However, the time required to perform a successful dissection without damage to the nerve is greater and cats are more expensive.

An alternative approach may be easily used in the rodent. The tail nerve is prepared as for motor nerve determinations, except that the cathode is placed proximally instead of distally at the two stimulation locations. The recording electrodes are placed on the skull for recording the somatosensory evoked potential. The somatosensory evoked potential represents activity along a number of tracts and across a number of synapses. However, the difference between the latency to the onset of the first peak of the response, when recorded following stimulation at two tail locations 40 mm apart should give an accurate measurement of the conduction time between the two stimulating electrodes and will reflect only sensory activity (Giblin 1980). The conduction velocity is calculated by dividing the distance between the two stimulating cathodes by the difference between the two latencies. Should the peak of the somatosensory response be so broad that it cannot be replicate with an accuracy of less than 5% of the latency difference observed, then a point on the rising phase of the potential should be chosen, e.g. at a voltage 50% of the peak voltage. Alternatively, the sensory nerve conduction velocity can be obtained from a purely sensory nerve or from stimulation of the dorsal rootlets of a mixed nerve, using two recording electrode pairs to obtain the conduction velocity by difference in the latency.

In using the somatosensory evoked potential to determine the sensory nerve conduction velocity, it is necessary to average more than 16 responses. A reasonable number of responses to average is between 64 and 128. The stimulation frequency must be slowed to about 0.5 hz for these studies since more rapid stimulation will alter the properties of the first wave of the somatosensory evoked potential. The electrodes for recording the somatosensory evoked potential may be either small stainless steel screws threaded into the skull (0-80 or 00-90), or electrode wires applied to the surface of the skull. If the latter approach is taken, care must be taken to ensure that good electrical contact is maintained between the skull and the electrode. An electrolyte gel applied in a small dab at the electrode tip is sufficient for this purpose. Precise electrode location is not critical as long as it is the same from one animal to the next. A useful configuration for this experiment in rats is to place the active electrode 2 mm posterior and 1 mm lateral to bregma on the side contralateral to the stimulation electrodes. The reference electrode may be place 5 mm anterior to bregma along the midline.

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MOTOR ACTIVITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
U.S. ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

A. General

In the assessment and evaluation of the toxic characteristics of a substance, determination of the effects of administration of the substance on motor activity is useful when neurotoxicity is suspected.

B. Acute Motor Activity Test

The purpose of the acute motor activity test is to examine changes in motor activity occurring over a range of acute exposure levels These changes may then be evaluated in the context of changes occurring in other organ systems. This test is an initial step in determining the potential of a substance to produce acute neurotoxicity and may be used to screen members of a class of substances for known neurotoxicity, and/or to establish a dosage regimen prior to the initiation of subchronic neurotoxicity testing.

C. Subchronic Motor Activity Test

The purpose of the subchronic motor activity test is to determine whether the repeated administration of a suspected neurotoxicant results in changes in motor activity. These changes may be evaluated in the context of changes occurring in other organ systems. This test is an initial step in determining the potential of a substance to produce subchronic neurotoxicity.

II. DEFINITIONS

A. Neurotoxicity is the adverse effect on the structure or function of the central and/or peripheral nervous system related to exposure to a chemical substance.

B. Motor activity is any movement of the experimental animal.

C. A toxic effect is an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

III. PRINCIPLE OF THE TEST METHOD

The test substance is administered to several groups of experimental animals, one dose being used per group. Measurements of motor activity are made. The exposure levels at which significant changes in motor activity are produced is compared to those levels which produce toxic effects not originating in the central and/or periperal nervous system.

IV. TEST PROCEDURES

A. Animal Selection

1. Species and Strain

Testing shall be performed in a laboratory rat or mouse. The choice of species should take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies, the potential for combined studies, and the availability of other toxicity data for the species.

2. Age

Young adult animals (at least 42 days old for rat or mouse) should be used.

3. Sex

a. Equal numbers of animals of each sex are required for each dose level for the motor activity test.

b. The females should be nulliparous and non-pregnant.

B. Number of Animals

Animals shall be randomly assigned to test and control groups. Each test or control group must be designed to contain a sufficient number of animals at the completion of the study to detect a 40% change in activity of the test groups relative to the control group with 90% power at the 5% level. For most designs, calculations can be made according to Dixon and Massey (1957), Neter and Wasserman (1974), Sokal and Rohlf (1969), or Jensen (1972).

C. Control Groups

1. A concurrent control group is required. This group must be an untreated group, or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

2. Positive control data are required to demonstrate the sensitivity and reliability of the activity measuring device and testing procedure. These data should demonstrate the ability to detect increases or decreases in activity and to generate a dose-effect curve or its equivalent using three values of the dose or equivalent independent variable. A single administration of the dose (or equivalent) is sufficient. It is recommended that chemical exposure be used to collect positive control data. Positive control data shall be collected at the time of the test study unless the laboratory can demonstrate the adequacy of historical data for this purpose.

3. A satellite group may be treated with the high dose level for 90 days and observed for reversibility, persistence or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days.

D. Dose Levels and Dose Selection

At least 3 doses, equally spaced on a log scale (e.g., 1/2 log units) over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve. The highest dose shall produce 1) clear effects on motor activity or 2) life-threatening toxicity. The data from the lower doses must show either 1) graded dose-dependent effects at 2 dose levels or 2) no effects at 2 dose levels, respectively.

E. Duration of Testing

The duration of exposure will be specifed in the test rule.

F. Route of Administration

The test substance shall be administered by the method specified in the test rule. This will usually be the route most closely approximating the route of human exposure. The exposure protocol shall conform to that outlined in the appropriate acute or subchronic toxicity study guideline.

G. Combined Protocol

The tests described herein may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination.

H. Study Conduct

1. General

Motor activity must be monitored by an automated activity recording apparatus. The device used must be capable of detecting both increases and decreases in activity, i.e. baseline activity as measured by the device must not be so low as to preclude decreases nor so high as to preclude increases. Each device shall be tested by a standard procedure to ensure, to the extent possible, reliability of operation across devices and across days for any one device. In addition, treatment groups must be balanced across devices. Each animal shall be tested individually. The test session shall be long enough for motor activity to approach asymptotic levels by the last 20% of the session for most treatments and animals. All sessions should have the same duration. Treatment groups shall be counterbalanced across test times. Effort should be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables which can affect motor activity are sound level, size and shape of the test cage, temperature, relative humidity, lighting conditions, odors, use of home cage or novel test cage and environmental distractions. Tests shall be executed by an appropriately trained individual.

2. Acute

Testing shall be timed to include the time of peak signs.

3. Subchronic

All animals shall be tested prior to initiation of exposure and at 30 ±2, 60 ±2 and 90 ±2 days during the exposure period. Testing shall occur prior to the daily exposure. Animals shall be weighed on each test day and at least once weekly during the exposure period.

V. DATA REPORTING AND EVALUATION

In addition to the reporting requirements specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the final test report must include the following information:

A. Description of System and Test Methods

1. Positive control data from the laboratory performing the test which demonstrate the sensitivity of the procedure being used.

2. Procedures for calibrating and assuring the equivalence of devices and balancing treatment groups.

B. Results

The following information must be arranged by test group (dose level).

1. In tabular form, data must be provided showing for each animal:
   - a. Its identification number;
   - b. Body weight, total session activity counts, and intrasession subtotals for each date measured.
2. Group summary data should also be reported.

C. Evaluation of Data

An evaluation of the test results (including statistical analysis comparing total activity counts at the end of exposure of treatment vs control animals must be made and supplied. This submission must include dose-effect curves for motor activity expressed as activity counts.

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HS-Neuro-Motor Act
October, 1983

MOTOR ACTIVITY

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
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TABLE OF CONTENTS

Pages

I. INTRODUCTION.. 1

II. RATIONALES FOR STUDY DESIGN......................... 1
  A. Species.. 1
  B. Age.. 2
  C. Number of Animals.............................. 2
  D. Dose Selection................................. 2
  E. Route of Exposure.............................. 2

III. RATIONALES FOR STUDY CONDUCT....................... 3
  A. Apparatus...................................... 3
  B. Time of Testing................................ 3
  C. Environmental Variables........................ 4
  D. Session Length................................. 4
  E. Frequency of Testing........................... 4
  F. Individual Measurement......................... 4

REFERENCES.. 10

I. INTRODUCTION

Motor activity has been extensively studied in both behavioral pharmacology and behavioral toxicology (Reiter 1978, Reiter and MacPhail 1979, Irwin 1968, Kinnard and Watzman 1966). There are several reasons for this popularity: 1. Motor activity occurs naturally. All animals, including man, explore their environment. 2. The functional state of the nervous system is often reflected in amounts of motor activity and represents behavior which is relevant to the animal's survival (Reiter 1978). 3. Studies of motor activity indicate that it is sensitive to the effects of a wide variety of agents (Dews 1953, Fibiger and Campbell 1971, Fibiger and Campbell 1971, Irwin 1968, Kellogg and Lundborg 1972, Norton et al. 1976, Segal 1975, Silverman and Williams 1975, Vasko et al. 1979, Waldeck, 1974, 1975, Ahlenius et al. 1973, Anden et al. 1973, Campbell and Mabry 1973, Costa et al. 1972, Creese and Iverson 1973, Erinoff et al. 1979, Foldes and Costa 1975, Jacobs et al. 1974, Sulser et al. 1968, Weissman et al. 1966). 4. Measurement of motor activity is relatively easy; no training or deprivation techniques are necessary. There are many commercially available devices which give a reliable quantification of motor activity (Reiter and MacPhail 1979, Kinnard and Watzman 1966, Robbins 1977).

II. RATIONALES FOR STUDY DESIGN

A. Species

Rodents have been widely used and extensively studied in behavioral pharmacology and toxicology. There are many devices capable of measuring the motor activity of rats and mice. The history of the development of psychoactive drugs indicates that the motor activity of rats and mice are predictive of psychoactive potential in humans (Irwin 1968, Kinnard and Watzman 1966, Dews 1953, Turner 1965). Because rats are the preferred rodent species specified in the subchronic toxicity guidelines, their use in behavioral studies will facilitate combined studies and aid in the integration of data. However, mice may be preferable, particularly if the metabolic and/or toxicity data indicate that they are a more appropriate choice and if considerable data exist on the chemical's effects in mice. More data are available on motor activity in mice. They are more easily handled, require less space, and in a subchronic study, the change of the size relationship of mouse to testing equipment is not as drastic as with rats. In addition mice are generally less costly. Since a sufficient data base has not been established for larger mammals, their use is discouraged.

B. Age

Practical limitations (i.e. weaning) preclude exposure before Day 21. For subchronic studies, exposure should not start later than approximately Day 42, corresponding to the subchronic toxicity guidelines. This includes the normal adult life span and is maximally cost effective.

C. Number of Animals

The sensitivity of the test will depend on both the group size and the normal variability of the test system (Sokal and Rohlf 1969). Baseline motor activity can be quite variable (a coefficient of variation of 25% is not uncommon). Thus, a group size of 10 will allow detection of about a 40% change in activity at the 5% level with 90% certainty. A group size greater than 34 would be required to detect a 20% change with the same confidence and certainly. For substances which affect motor activity, a 40% change will be adequate for determining whether the dose range which affects activity is well below the generally toxic range.

D. Dose Selection

Many, if not most chemicals, administered at fatal or maximal doses will have serious behavioral effects. Yet effects at these levels may not be indicative of a graded relationship between exposure and behavior by any specific mechanism. Behavioral effects may not occur at lower doses, or the effects observed at lower doses may be qualitatively different from those observed at high doses (e.g. stimulant at low soses and depressant at high doses).

Dews (1972) discusses some of these and other reasons which underscore the importance of dose-effect relationships collected over a range of doses. He recommends (and we endorse) separation of doses by multiples of 3 (1/2 log units) over a 10-30 fold range.

E. Route of Exposure

EPA is generally requiring that test substances be administered by the route that duplicates or most closely simulates the major known or expected route by which human exposure occurs. This is the accepted method because results are generally directly amenable to evaluation in terms of potential human health hazards.

However, if humans are exposed via several routes, the major route of exposure may not be the most important determinant. In this case, EPA may consider the most important determinant to be the route which is anticipated to be the most sensitive in terms of repeated exposure toxicity. In deciding on route of expousre, EPA will consider not only human use or exposure but also specific properties of the chemical including its absorption, distribution and metabolism.

III. RATIONALES FOR STUDY CONDUCT

A. Apparatus

The use of automated activity measurement devices is proposed. These devices have been widely used and validated and are more readily standardized and cheaper to perform than subjective rating scales. (Reiter and MacPhail 1979, Robbins 1977, Kinnard and Watzmann 1966). The use of commercially available devices is encouraged. It is important that an activity-measuring device detects both increases and decreases in activity should they occur; some tilt-cages (stabilimeters) engender a low baseline activity such that decreases would be difficult to detect. It should be noted that many types of field detector devices are relatively new and have not been subjected to as wide a validation. However their major problem has been the recording of many types of activity rather than specifically locomotor activity (Reiter and MacPhail 1979). While this is a problem for students of locomotion per se, it is less so when the major objective is toxicity screening (Reiter and MacPhail 1979). The Agency sees no reason to prohibit the use of such devices as long as differences in operational characteristics can be controlled for.

B. Time of Testing

The time of testing with respect to the light-dark cycle is an important variable that influences activity (Robbins 1977). Locomotor activity is subject to control by an apparently endogenous rhythm, the precise hormonal and neurochemical correlates of which are still largely unknown (Reinberg and Halberg 1971). Many contradictory findings on activity levels after drug and chemical exposures might be due to a lack of standardization of this factor. Therefore, the guideline specifies that treatment groups must be balanced across test times.

During repeated exposure, testing shall occur before exposure to eliminate acute effects of the test substance.

C. Environmental Variables

Many environmental variables affect motor activity. Poor control of these variables will result in increased control group variability, thus necessitating larger sample sizes. The variables listed in the guideline are among those that should be controlled to ensure the reliability of the testing procedure.

D. Session Length

Motor activity is typically very high when the animal first enters the test environment and decreases with time. To minimize the influence of presession handling and other external variables, the session should be long enough for motor activity to have stabilized. This will also tend to equalize the measurements across different types of apparatus, which may differ with respect to the time for such stability to be reached.

E. Frequency of Testing

For repeated exposure protocols, monthly testing is proposed as a way to obtain data on the effects of duration of exposure without excessive investment of facilities or labor. Animals should be tested before the initial exposure to obtain a baseline level. This will provide a check on the randomization of treatment groups with respect to activity.

F. Individual Measurement

Motor activity and the effects of chemical agents on such activity may differ when animals are tested in groups rather than individually (Watzman et al 1966, Reiter 1978). For purposes of toxicity screening, the variability induced by group measurement would complicate the interpretation of the data.

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HG-Neuro-Acute Delayed
October, 1983

ACUTE DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS SUBSTANCES

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
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I. PURPOSE

Organophosphorus substances should be considered as candidates for delayed neurotoxicity studies using the adult hen as the test animal. This test has certain limitations, e.g. in predicting effects from repeated exposures. These limitations may be minimized by conducting an adjunct test in which inhibition and aging of neurotoxic esterase of hen neural tissue are measured.

II. DEFINITIONS

Acute delayed neurotoxicity is a prolonged, delayed-onset locomotor ataxia resulting from single administration of the test substance, repeated once if necessary.

III. PRINCIPLE OF THE TEST METHOD

The test substance is administered orally in a single dose to domestic hens (Gallus gallus domesticus) which have been protected from acute cholinergic effects, when appropriate. The animals are observed for at least 21 days for delayed neurotoxicity, with redosing and observation for another 21 days if no effects or equivocal responses are seen. The animals are observed daily for behavioral abnormalities, locomotor ataxia and paralysis. Histopathological examination of selected neural tissues is undertaken on all animals surviving the initial cholinergic phases.

IV. TEST PROCEDURES

A. Animal Selection

The adult domestic laying hen, aged between 8-14 months, is recommended. Standard size breeds and strains should be employed.

B. Number of Animals

A sufficient number of hens should be utilized so that at least six survive the observation period.

C. Control Groups

1. General

Appropriate control groups should be used. These should include a positive control group of at least two hens treated with a known delayed neurotoxicant and a concurrent control group of at least six hens treated in a manner identical to the treated group, except that administration of the test substance and any protective agents is omitted.

2. Reference Substances

A substance which is known to produce acute delayed neurotoxicity should be used as a positive control. Examples of such substances are triorthocresyl phosphate (TOCP) and leptophos.

D. Housing and Feeding Conditions

Cages or enclosures which are large enough to permit free mobility of the hens and easy observation of gait should be used. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Appropriate diets should be administered as well as an unlimited supply of drinking water.

E. Dose Level

The selected dose level of the test substance should not be less than the unprotected LD50 dose. Atropine or another non-interfering protective agent may be used to prevent death due to acute cholinergic effects. Doses of test substances higher than 5000 mg/kg of body weight need not be tested.

F. Dose Selection

A preliminary LD50 test using an appropriate number of animals, dosages and dose groups, as recommended in Test Guideline HG-Acute-Oral, should be performed in unprotected hens to establish the dose level to be used in this test. Healthy young adult hens free from interfering viral diseases and medication and without abnormalities of gait should be acclimatized to the laboratory conditions for at least five days prior to randomization and assignment to treatment and control groups.

G. Route of Administration

Dosing with the test substance should normally be by the oral route using gavage, gelatine capsules, or a comparable method.

H. Study Conduct

1. General

The test or control substance should be administered and observations begun. All hens should be carefully observed at least once daily for a period of at least 21 days and signs of toxicity recorded, including the time of onset, degree and duration. Observations should include, but not be limited to, behavioral abnormality, locomotor ataxia and paralysis. At least twice a week the hens should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to enhance the observation of minimal responses. If neurotoxic responses are not observed or if equivocal responses are seen, then the dose should be administered again and the animals observed for an additional 21 days. The hens should be weighed weekly. Any moribund hens should be removed and sacrificed.

2. Pathology

a. Gross Necropsy

In the presence of clinical signs of delayed neurotoxicity useful information may be provided by gross necropsy.

b. Histopathology

All animals should be subjected to microscopic examination. Tissues should be fixed *in situ*, preferably using perfusion techniques. Sections should include medulla oblongata, spinal cord and peripheral nerves. The spinal cord sections should be taken from the upper cervical bulb, the midthoracic and the lumbo-sacral regions.

Section of the proximal region of the tibial nerve and its branches should be taken. Sections should be stained with appropriate myelin and axon-specific stains.

V. DATA REPORTING AND EVALUATION

A. Test Report

In addition to the reporting requirements specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the final test report must include the following information:

1. Toxic response data by group with a description of clinical manifestations of nervous system damage; where a grading system is used the criteria should be defined;
2. For each animal, time of death during the study or whether it surivived to termination;
3. The day of observation of each abnormal sign and its subsequent course;
4. Body weight data;
5. Necropsy findings for each animal, when performed;
6. A detailed description of all histopathological findings;
7. Statistical treatment of results, where appropriate.

B. Treatment of Results

Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions or effects, the types of lesions or effects and the percentage of animals displaying each type of lesion or effect.

C. Evaluation of Results

The findings of an acute delayed neurotoxicity study should be evaluated in terms of the incidence and severity of neurotoxic effects and of any other observed effects and histopathological findings in the treated and control groups.

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HG-Neuro-Subchronic Delayed
October, 1983

SUBCHRONIC DELAYED NEUROTOXICITY OF ORGANOPHOSPHORUS SUBSTANCES

OFFICE OF TOXIC SUBSTANCES
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HG-Neuro-Subchronic Delayed
October, 1983

I. PURPOSE

In the assessment and evaluation of the toxic characteristics of organophosphorus substances the determination of subchronic delayed neurotoxicity may be carried out, usually after initial information on delayed neurotoxicity has been obtained by acute testing or by the demonstration of inhibition and aging of neurotoxic esterase in hen neural tissue. The subchronic delayed neurotoxicity test provides information on possible health hazards likely to arise from repeated exposures over a limited period of time. It will provide information on dose response and can provide an estimate of a no-effect level which can be of use for establishing safety criteria for exposure.

II. DEFINITIONS

Subchronic delayed neurotoxicity is a prolonged, delayed-onset locomoter ataxia resulting from repeated daily administration of the test substance.

III. PRINCIPLE OF THE TEST METHOD

Multiple dose levels of the test substance are administered orally to domestic hens (Gallus gallus domesticus) for 90 days. The animals are observed at least daily for behavioral abnormalities, locomotor ataxia and paralysis. Histopathological examination of selected neural tissues is undertaken at the termination of the test period.

IV. TEST PROCEDURES

A. Animal Selection

The adult domestic laying hen, aged between 8-14 months, is recommended. Standard size breeds and strains should be employed.

B. Number of Animals

Ten hens should be used for each treatment and control group.

C. Control Group

1. General

A concurrent control group should be used. This group should be treated in a manner identical to the treated group, except that administration of the test substance is omitted.

2. Reference Substances

If a positive control is used, a substance which is known to produce delayed neurotoxicity should be employed. Examples of such substances are triorthocresyl phosphate (TOCP) and leptophos.

D. Housing and Feeding Conditions

Cages or enclosures which are large enough to permit free mobility of the hens and easy observation of gait should be used. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Appropriate diets should be administered as well as an unlimited supply of drinking water.

E. Dose Levels

At least three dose levels should be used in addition to the control group(s). The highest dose level should result in toxic effects, preferably delayed neurotoxicity, but not produce an incidence of fatalities which would prevent a meaningful evaluation. The lowest dose level should not produce any evidence of toxicity.

F. Route of Administration

Oral dosing each day for at least five days per week should be carried out, preferably by gavage or administration of gelatine capsules.

G. Study Conduct

1. General

Healthy young adult hens free from interfering viral diseases and medication and without abnormalities of gait should be acclimatized to the laboratory conditions for at least five days prior to randomization and assignment to treatment and control groups. The test or control substance should be administered and observations begun. All hens should be carefully observed at least once daily throughout the test period. Signs of toxicity should be recorded, including the time of onset, degree and duration. Observations should include, but not be limited to, behavioral abnormality, locomotor ataxia and paralysis. At least once a week the hens should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to enhance the observation of minimal responses. The hens should be weighed weekly. Any moribund hens should be removed and sacrificed.

2. Pathology

a. Gross Necropsy

In the presence of clinical signs of delayed neurotoxicity useful information may be provided by gross necropsy.

b. Histopathology

Tissues from all animals should be fixed *in situ*, using perfusion techniques. Sections should include medulla oblongata, spinal cord and peripheral nerves. The spinal cord sections should be taken from the upper cervical bulb, the mid-thoracic and lumbosacral regions. Sections of the proximal region of the tibial nerve and its branches and of the sciatic nerve should be taken. Sections should be stained with appropriate myelin and axon-specific stains. Microscopic examination should be carried out on all hens in the control and high-dose groups. Microscopic examination should also be carried out on hens in the low and intermediate dose groups when there is evidence of effects in the high-dose group.

V. DATA REPORTING AND EVALUATION

A. Test Report

In addition to the reporting requirements specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the final test report must include the following information:

1. Toxic response data by group with a description of clinical manifestations of nervous system damage; where a grading system is used the criteria should be defined;
2. For each animal, time of death during the study or whether it survived to termination;
3. The day of observation of each abnormal sign and its subsequent course;
4. Body weight data;
5. Necropsy findings for each animal, when performed;
6. A detailed description of all histopathological findings;
7. Statistical treatment of results, where appropriate.

B. Treatment of Results

Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions or effects, the types of lesions or effects and the percentage of animals displaying each type of lesion or effect.

All observed results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods should be selected during the design of the study.

C. Evaluation of Results

The findings of a subchronic delayed neurotoxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the incidence and severity of observed neurotoxic effects and any other observed effects and histopathological findings in the treated and control groups. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level based on lack of clinical signs and histopathological changes.

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HG-Neuro-Battery
October, 1983

FUNCTIONAL OBSERVATIONAL
BATTERY

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I. PURPOSE

In the assessment and evaluation of the potential human health effects of substances, it may be necessary to test for neurotoxic effects. Substances that have been observed to cause neurotoxic signs (e.g., convulsions, tremors, ataxia) in other toxicity tests, as well as those having a structural similarity to known neurotoxicants, should be evaluated for neurotoxicity. The functional observational battery is a noninvasive procedure designed to detect gross functional deficits in young adults resulting from exposure to chemicals and to better quantify neurotoxic effects detected in other studies. This battery of tests is not intended to provide a detailed evaluation of neurotoxicity. It is designed to be used in conjunction with neuropathologic evaluation and/or general toxicity testing. Additional functional tests may be necessary to assess completely the neurotoxic potential of a chemical.

II. DEFINITIONS

A. Neurotoxicity is any adverse effect on the structure or function of the central and/or peripheral nervous system related to exposure to a chemical substance.

B. A toxic effect is an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

III. PRINCIPLE OF THE TEST METHOD

The material is administered by an appropriate route to laboratory rodents. The animals are observed under carefully standardized conditions with sufficient frequency to ensure the detection of behavioral and/or neurologic abnormalities, if present. Various functions that could be affected by neurotoxicants are assessed during each observation period.

IV. TEST PROCEDURES

A. Animal Selection

1. Species and Strain

The laboratory rat or mouse is recommended. Although information will generally be lacking, whenever possible the choice of species should take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies. The potential for combined studies should also be considered. Standard strains should be used.

2. Age

Young adult animals (at least 42 days old for the rat or mouse) should be used.

3. Sex

a. Equal numbers of animals of each sex are required for each dose level.

b. The females should be nulliparous and nonpregnant.

B. Number of Animals

All exposed animals should be tested. At least 10 animals of each sex should be used at each dose level. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the end of the study. Animals should be randomly assigned to treatment and control groups.

C. Control Groups

1. A concurrent ("sham" exposure or vehicle) control group is required Subjects should be treated in the same way as for an exposure group except that administration of the test substance is omitted.

2. Concurrent or historic data from the laboratory performing the testing should provide evidence of the ability of the procedures used to detect major neurotoxic endpoints such as limb weakness or paralysis (e.g., acrylamide), CNS stimulation (e.g., β,β'-iminodiproprionitrile) autonomic signs (e.g., physostigmine).

3. A satellite group may be treated with the high dose level for the duration of exposure and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate duration, normally not less than 28 days.

D. Dose Levels and Dose Selection.

At least 3 doses, equally spaced on a log scale (e.g., 1/2 log units) over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve. The highest dose shall produce 1) clear behavioral effects or 2) life-threatening toxicity. The data from the lower doses must show either 1) graded dose-dependent effects at 2 dose levels or 2) no effects at 2 dose levels, respectively.

E. Duration and Frequency of Exposure

The duration and frequency of exposure will be specified in the test rule.

F. Route of Exposure

The test substance shall be administered by the route specified in the test rule. This route will usually be the one most closely approximating the expected route of human exposure. The exposure protocol shall conform to that outlined in the appropriate acute or subchronic toxicity study guideline (USEPA 1982).

G. Combined Protocol

Subjects used for other toxicity studies may be used if none of the requirements of either study are violated by the combination.

H. Study Conduct

All animals in a given study should be observed carefully by the same trained technician who is blind with respect to the animals' treatments. All animals should be observed prior to initiation of exposure. Subsequent observations should be made with sufficient frequency to ensure the detection of behavioral and/or neurologic abnormalities, if present. At minimum, observations at 1 hour, 6 hours, 24 hours, 7 days, and 14 days and monthly thereafter are recommended. In a subchronic study, subsequent to the first exposure, all observations should be made before the daily exposure. The animals should be removed from the home cage to a standard arena for observation. Effort should be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables that can affect behavior are sound level, temperature, humidity, lighting, odors, time of day, and environmental distractions. Explicit, operationally defined, scales for each function should be used. The development of objective quantitative measures of the observational endpoints specified is encouraged.

The following is a minimal list of observations that should be noted:

1. Any unusual responses with respect to body position, activity level, coordination of movement, and gait;

2. Any unusual or bizarre behavior including, but not limited to, headflicking, head searching, compulsive biting or licking, self-mutilation, circling, and walking backwards;

3. The presence of:

   (a) Convulsions;

   (b) Tremors;

   (c) Increased levels of lacrimation and/or red-colored tears;

   (d) Increased levels of salivation;

   (e) Piloerection;

(f) Pupillary dilation or constriction;

(g) Unusual respiration (shallow, labored, dyspneic, gasping, and retching) and/or mouth breathing;

(h) Diarrhea;

(i) Excessive or diminished urination; and

(j) Vocalization.

4. Forelimb/hindlimb grip strength. The procedure described by Meyer et al. (1979) is recommended.

5. Sensory function. A simple assessment of sensory function (vision, audition, pain perception) should be made. Marshall et al. (1971) have described a neurologic exam for this purpose; these procedures are also discussed by Deuel (1977). Irwin (1968) described a number of reflex tests intended to detect gross sensory deficits, including the visual placing response, Preyer reflex, and tail pinch. Many procedures have been developed for assessing pain perception (e.g., Ankier 1974; D'Amour and Smith 1941; Evans 1971).

V. DATA REPORTING AND EVALUATION

In addition to the reporting requirements specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations], the final test report must include the following information.

A. Description of System and Test Methods

1. A detailed description of the procedures used to standardize observation, including the arena and operational definitions for scoring observations.

2. Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. Historic data may be used if all aspects of the experimental protocol are the same, including personnel.

B. Results

The following information must be arranged by test group dose level.

1. In tabular form, data for each animal must be provided showing:
    a. Its identification number; and
    b. Its body weight and score on each sign at each observation time, the time and cause of death (if appropriate).
2. Summary data for each group must include:
    a. The number of animals at the start of the test;
    b. The number of animals showing each observation score at each observation time;
    c. The percentage of animals showing each abnormal sign at each observation time; and
    d. The mean and standard deviation for each continuous endpoint at each observation time.

C. Evaluation of Data

The findings of a functional observational battery should be evaluated in the context of preceding and/or concurrent toxicity studies and any correlative histopathological findings. The evaluation should include the relationship between the doses of the test substance and the presence or absence, incidence and severity, of any neurotoxic effects. The evaluation should include appropriate statistical analyses. Choice of analyses should consider tests appropriate to the experimental design and needed adjustments for multiple comparisons.

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October, 1983

FUNCTIONAL OBSERVATIONAL BATTERY

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TABLE OF CONTENTS

		PAGE
I.	INTRODUCTION	1
II.	RATIONALE FOR STUDY DESIGN	2
	A. Species	2
	B. Age	2
	C. Number of Animals	2
	D. Dose Selection	3
	E. Satellite Group	3
	F. Route of Exposure	3
III.	RATIONALE FOR STUDY CONDUCT	4
	A. Endpoints Selected	4
	B. Training of Observer	4
	C. Frequency and Time of Observation	5
	D. Environmental Variables	5
	E. Operational Definitions and Scales of Measurement	5
REFERENCES		6

I. INTRODUCTION

Observations of animals are called for in certain sections of the Test Standards for Acute and Subchronic Health Effects. Among the signs of toxicity to be observed daily are behavioral signs; yet no suggestions for what behaviors to observe or how to score the data are provided. Using this functional observational battery at selected times during treatment will serve to meet the intent of the Test Standards for Acute and Subchronic Health Effects. This type of behavioral testing should be combined routinely with the testing for other health effects.

The range of complexity of techniques for neurotoxicity assessment is matched only by the range of complexity of functions governed by the nervous system. The functional observational battery is designed to apply a low level of technical complexity to a range of behaviors indicative of neurologic function. Such a battery should "screen" chemical agents for which there is little information on potential sites of action but that are suspect neurotoxicants. The battery may identify neurotoxic endpoints and thus suggest more complex, focused evaluation upon which risk assessments may be based. It should aid in evaluating the significance of morphologic changes. Absence of both functional and morphologic changes provide some assurance that an unstudied agent does not have a high potential for causing neurotoxic damage.

Ideally, a neurotoxicity screening battery should be capable of detecting deficits in the major functions governed by the nervous system: sensation, movement, cognition, emotion, and life maintenance. In reality, simple techniques for assessing sensory and cognitive deficits are nonexistent. Thus, the battery is limited to observation of autonomic signs, general indicators of behavior and motor function. Reflex techniques for assessing vision and audition are included but will only detect almost total blindness or deafness. The inclusion of sensitive and objective measures of vision, audition and cognitive functions would entail a large increase in complexity and expense not warranted for initial evaluation of a chemical's neurotoxic potential. When available data indicate that an agent may cause deficits in these areas, a more complete analysis should be done using an appropriate animal model.

Behaviors may change as a result of damage to other organ systems than the nervous system. It is important to evaluate the results of a behavioral screen in context with the results of other toxicity testing.

II. RATIONALE FOR STUDY DESIGN

A. Species

Rodents have been widely used and extensively studied in behavioral pharmacology and toxicology. The history of the development of psychoactive drugs indicates that the behavior of rats and mice is predictive of psychoactive potential in humans (Irwin 1968, Kinnard and Watzman 1966, Turner 1965). Because rats are the preferred rodent species for subchronic toxicity testing, their use for neurotoxicity screening will facilitate combined studies and aid in the integration of data.

B. Age

Because considerable neurologic development occurs during the neonatal period, exposure and neurotoxicity screening should not start before day 42. This includes the normal adult life span, is cost effective, and reduces the confounding effects of rapid development in the early weeks of life.

There may be situations in which it is desirable to evaluate neurobehavioral development. When such situations arise, specific guidance will be provided on a case-by-case basis.

C. Number of Animals

The minimum number of animals specified (10 males and 10 females per treatment group) conforms to the recommendation for subchronic toxicity testing (USEPA 1982). This number will allow the functional observational battery to be done in conjunction with other toxicity testing. The nature of most of the data obtained from the observations outlined requires categorical or nonparametric statistical evaluation (Siegel 1956; Gad 1982; Gad and Weil 1982). The latter statistical procedures are generally less powerful than parametric tests when enough is known to specify a parametric test. In any case, the larger the number of animals examined, the greater the confidence that an adverse effect will not be missed.

D. Dose Selection

Many, if not most chemicals, administered at fatal or maximal doses will have serious behavioral effects. Yet effects at these levels may not be indicative of a graded relationship between exposure and behavior by any specific mechanism. Behavioral effects may not occur at lower doses, or the effects observed at lower doses may be qualitatively different from those observed at high doses (e.g. stimulant at low doses and depressant at high doses).

Dews (1972) discusses some of these and other reasons which underscore the importance of dose-effect relationships collected over a range of doses. He recommends (and we endorse) separation of doses by multiples of 3 (1/2 log units) over a 10-30 fold range.

E. Satellite Group

The suggestion for a satellite group was adopted from the OECD guidelines (1981). It was felt that 28 days would be sufficient to allow observation of delayed toxicity and reversibility of early onset toxic effects.

F. Route of Exposure

EPA generally requires that test substances be administered by the route that duplicates or most closely simulates the major known, or expected, route by which human exposure occurs to facilitate evaluation in terms of potential human health hazards. However, if humans are to be exposed by several routes, the major route may not be the most important determinant. The EPA may consider the most important determinant to be the route that is anticipated to be the most harmful in terms of repeated exposure toxicity. Therefore, in deciding on route of exposure, the EPA will consider specific properties of the chemical, including its absorption, distribution and metabolism.

III. RATIONALE FOR STUDY CONDUCT

A. Endpoints Selected

The major objective of this guideline is to ensure that appropriate attention is paid to the observational procedures that will detect neurotoxicity. The endpoints selected have been amassed from reports of observed signs from studies of neurotoxic agents. The signs most frequently observed include changes in arousal (hyperactivity, hyperreactivity, and lethargy), other signs of CNS stimulation (convulsions and tremors), limb weakness or paralysis, autonomic nervous system activity and sensory disturbances (Spencer and Schaumberg 1980). Thus, the history of neurotoxicology in humans and animals confirms the validity of the endpoints chosen.

B. Training of Observer

The quality of data obtained is primarily dependent upon the quality of the observer. Careful training and quality controls are essential to assure reliability. Over a period of time, changes in procedures will undoubtedly occur unless precautions are undertaken. Irwin (1966) recommends that several precautions be taken including the following:

1. Monitoring among observers with immediate feedback when deviations of technique are noted;

2. Independent data accumulation by two observers, the data accepted when good agreement exists and immediate inquiry initiated when divergence appears;

3. "Blind" testing of a standard compound at approximately two-month intervals for test-retest reliability control;

4. Assessment of the technique of the observer at monthly intervals; and

5. Review of instructional materials at approximately two-month intervals.

C. Frequency and Time of Observation

Observation should be made with sufficient frequency to detect behavioral and neurologic signs as they develop. As an example, for acute studies, Gad (1982) observes at 1 hour, 6 hours, 24 hours, 7 days, and 14 days after exposure. For a repeated exposure study, he performs his screen at 1 hour, 1 day, 7 days, 14 days, 30 days, and once a month thereafter. However, other schedules may also be appropriate and, if signs are observed, more frequent observation may be necessary to describe adequately the nature and time-course of the neurotoxic syndrome.

During repeated exposure, testing should occur before the daily exposure to minimize acute effects of the test substance. Observations should be done at the same part of the light/dark cycle as this is an important determinant of rodent behavior.

D. Environmental Variables

Many environmental variables affect behavior. Poor control of these variables will result in increased control group variability and decrease the power of the observations and measurements made. The variables listed in the guideline are among those that should be controlled to ensure the reliability of the testing procedure.

E. Operational Definitions and Scales of Measurement

An inherent difficulty with all-or-none measures is that they have low power to identify true effects as statistically significant with relatively small sample sizes. The Agency encourages the development of objective continuous measures of the observational endpoints specified in this guideline. In the absence of such measures, the power to detect differences may be increased by using explicit, operationally defined ordinal scales for each endpoint. Examples of operationally defined rating scales may be found in Irwin (1968) and Burkhalter and Balster (1979).

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HG-Neuro-Operant Behavior
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SCHEDULE-CONTROLLED OPERANT BEHAVIOR

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
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TABLE OF CONTENTS

PAGE

I. PURPOSE 1

II. DEFINITIONS 1

- A. Neurotoxicity, Behavioral Toxicity
- B. Operant, Operant Behavior, Operant Conditioning
- C. Schedule of Reinforcement

III. PRINCIPLE OF THE TEST METHOD 2

IV. TEST PROCEDURES 3

- A. Experimental Design
- B. Animal Selection
  - 1. Species
  - 2. Age
  - 3. Sex
  - 4. Experimental Hisotry
- C. Number of Animals
- D. Control Groups
  - 1. Vehicle Control
  - 2. Positive Control
- E. Dose Levels and Dose Selection
- F. Duration of Exposure
- G. Route of Administration
- H. Study Conduct
  - 1. General
  - 2. Apparatus
  - 3. Chamber Assignment
  - 4. Deprivation and Training
  - 5. Time, Frequency, and Duration of Testing
  - 6. Schedule Selection

V. DATA REPORTING AND EVALUATON 8

- A. Description of System, Test Methods, Experimental Design, and Control Data
- B. Results

REFERENCES 10

I. Purpose

In the assessment and evaluation of the potential human health effects of substances, it may be necessary to test for functional neurotoxic effects. Substances that have been observed to produce neurotoxic signs in other toxicity studies (e.g. CNS depression or stimulation), as well as substances with a structural similarity to known neurotoxicants should be evaluated for these effects.

This guideline defines procedures for conducting studies of schedule-controlled operant behavior, one way of evaluating functional neurotoxic effects (Dews, 1972; NAS 1975, 1977, 1982). Our purpose is to evaluate the effects of acute and repeated exposures on the rate and pattern of responding under schedules of reinforcement. Operant behavior tests may be used to evaluate many other aspects of behavior (Laties, 1978). Additional tests may be necessary to completely assess the behavioral effects of any substance. Behavioral evaluation should be used in conjunction with neuropathologic evaluation and the evaluation of other toxic effects.

II. Definitions

A. Neurotoxicity

Neurotoxicity or a neurotoxic effect is an adverse change in the structure or function of the nervous sytem following exposure to a chemical agent. Behavioral toxicity is an adverse change in the functioning of the organism with respect to its environment following exposure to a chemical agent.

B. Operant, Operant Behavior, Operant Conditioning

An operant is a class of behavioral responses which change or operates on the environment in the same way. Operant behavior is further distinguished as behavior which is modified by its consequences. Operant conditioning is the experimental procedure used to modify some class of behavior by reinforcement or punishment.

C. Schedule of Reinforcement

A schedule of reinforcement specifies the relation between behavioral responses and the delivery of reinforcers, such as food or water (Ferster and Skinner, 1957). For example, a fixed ratio (FR) schedule requires a fixed number of responses to produce a reinforcer (e.g. FR 30). On a fixed interval (FI) schedule, the first response after a fixed period of time is reinforced (e.g. FI 5 minutes).

III. Principle of the Test Method

Experimental animals are trained to perform under a schedule of reinforcement and measurements of their operant behavior are made. Several doses of the test substance are then administered according to the experimental design (between groups or within subjects) and the duration of exposure (acute or repeated). Measurements of the operant behavior are repeated. A descriptive and statistical evaluation of the data is made to evaluate the nature and extent of any changes in behavior in relation to exposures to the test substance. Comparisons are made between any exposures that influence the behavior and exposures that have neuropathological effects or effects on other targets of the chemical.

IV. Test Procedures

A. Experimental Design

These test procedures may be used to evaluate the behavior of experimental animals receiving either acute or repeated exposures. For acute exposure studies, either within-subject or between groups experimental designs may be used. For repeated exposure studies, between groups designs should be used, but within subject comparisons (pre-exposure and post-exposure) are recommended and encouraged.

B. Animal Selection

1. Species

For most studies, the laboratory mouse or rat is recommended. Standard strains should be used.

Under some circumstances other species may be recommended.

2. Age

Experimental animals should be young adults. Rats or mice should be at least 14 and 6 weeks old, respectively, prior to exposure.

3. Sex

a. Approximately equal numbers of male and female animals are required for each dose level and control group.

b. Virgin females should be used.

4. Experimental History

Animals should be experimentally and chemically naive.

C. Number of Animals

Six to twelve animals should be exposed to each level of the test substance and/or control procedure. If post-exposure effects are examined, a separate group, 6-12 additional animals not sacrificed for pathology, will be required in subchronic studies.

D. Control Groups

1. Untreated Controls

A concurrent "sham" exposure or vehicle control group or session (according to the design of the study) is required. The subjects should be treated similarly except that administration of the test substance is omitted.

2. Positive Controls

Positive control data is required to demonstrate that the experimental procedures, under the specific conditions in the testing laboratory, are sensitive to substances known to affect operant behavior. Both increases and decreases in response rate should be demonstrated. Data based on acute exposures will be adequate. Data should be collected according to the same experimental design as that proposed for the test substance. Historical data on the procedure collected in the same species and under the same conditions in the testing laboratory may be acceptable, but the presentation of concurrent control data is strongly encouraged since it provides evidence that the test has remained sensitive.

E. Dose Levels and Dose Selection

At least 3 doses, equally spaced over a log scale (e.g. 10, 30, 100), over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve. The highest dose shall produce: 1) clear behavioral effects; or 2) life-threatening toxicity. The data from the lower doses must show either: 1) graded dose-dependent effects at 2 dose levels; or 2) no effects at 2 dose levels, respectively.

F. Duration of Exposure

The duration and frequency of exposure will be specified in the test rule.

G. Route of Administration

The route of administration will also be specified in the test rule and will usually be identical to one of the anticipated or actual routes of human exposure. For some chemicals, another route (e.g. parenteral) may be justified. The exposure protocol should conform to that outlined in the appropriate acute or subchronic toxicity study guideline (USEPA, 1982).

H. Study Conduct

1. Apparatus

Behavioral responses and the delivery of reinforcers shall be controlled and monitored by automated equipment located so that its operation does not provide unintended cues or otherwise interfere with the ongoing behavior. Individual chambers should be sound attenuated to prevent disruptions of behavior by external noise. The response manipulanda, feeders, and any stimulus devices should be tested before each session; these devices should periodically be calibrated.

2. Chamber Assignment

Concurrent treatment groups should be balanced across chambers. Each subject should be tested in the chamber to which it is initially assigned.

3. Deprivation and Training

If a non-preferred positive reinforcer is used, all subjects should be deprived of food until they reach a fixed percentage (e.g. 80-90%, commonly) of their ad libitum body weight or for a fixed period (e.g., 18 hours) prior to training. Deprivation should be kept constant throughout the study.

Subjects must be trained until they display demonstrable stability in performance across days prior to exposure. One simple and useful criterion is a minimum number of sessions on the schedule and no systematic trend during the five days before exposure.

Cumulative records of cumulative responding over time for each animal should be presented to demonstrate that the pattern of responding is representative of that generated by the schedule of reinforcement.

4. Time, Frequency, and Duration of Testing

a. Time of Testing

All experimental animals should be tested at the same time of day and with respect to the time of exposure. For acute studies, testing should be performed when effects are estimated to peak, usually shortly after exposure. For subchronic studies, subjects should be tested prior to daily exposure in order to assess cumulative effects.

b. Frequency of Testing

The maintenance of stable operant behavior normally will require regular and frequent (e.g., 5 days a week) testing sessions. Animals should be weighed on each test day.

c. Duration of Testing

Experimental sessions should be long enough to reasonably see the effects of exposure, but brief enough to be practical. Under most circumstances, a session length of 30-40 minutes should be adequate.

If the nature or duration of effects following cessation of repeated exposure are a concern, animals from the high dose group should be tested following exposure for a suitable period of time.

5. Schedule Selection

The schedule of reinforcement chosen should generate response rates that may increase or decrease as a function of exposure. Many schedules of reinforcement can do this: a single schedule maintaining a moderate response rate; fixed-interval schedules, which engender a variety of response rates in each interval; or multiple schedules, where different components may maintain high and low response rates.

V. Data Reporting and Evaluation

In addition to the reporting requirements specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the final test report should contain the following information:

A. Description of System, Test Methods, Experimental Design, and Control Data

1. A description of the experimental chamber, programming equipment, data collection devices, and environmental conditions;

2. A description of the experimental design including counterbalancing procedures, and the stability criterion;

3. A description and statistical evaluation of positive control and other control data, including standard measures of central tendency, variability, coefficient of variation of response rates, and the slope of the dose-effect curve.

B. Results

1. Data for each animal should be arranged by test group in tabular form including the animal identification number, body weight, pre-exposure rate of responding, changes in response rate produced by the chemical, and group data for the same variables, including standard measures of central tendency, variability and coefficient of variation.

2. A description and statistical evaluation of the test results: with particular reference to the overall statistical procedures (e.g. parametric or non-parametric) dose-effect curve, and calculation of slope. Presentation of calculations is encouraged.

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SCHEDULE-CONTROLLED OPERANT BEHAVIOR

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TABLE OF CONTENTS

Page

I. INTRODUCTION 1

II. RATIONALE FOR STUDY DESIGN 2

A. Experimental Design
B. Species, Age, Sex
C. Number of Animals
D. Positive Controls
E. Dose Levels and Dose Selection

III. RATIONALE FOR STUDY CONDUCT 4

A. General
B. Chamber Assignment
C. Duration of Testing: Post-Exposure Effects
D. Schedule Selection

REFERENCES 6

I. INTRODUCTION

Schedule-controlled operant behavior has been widely used in both behavioral pharmacology (Thompson and Schuster, 1968; Harvey, 1971; McMillan and Leander, 1976; Weiss and Laties, 1976) and, more recently, in behavioral toxicology (Weiss and Laties, 1975; Geller et al., 1979). It is used by all of the Federal laboratories concerned with behavioral effects of chemicals and drugs (EPA, NIEHS, NIOSH, and FDA) and its use has been endorsed by 3 committees of the National Academy of Sciences (NAS, 1975 for EPA; NAS, 1977 for CPSC; NAS, 1982 for NTP).

There are many reasons why schedule-controlled operant behavior is both heuristically and empirically valuable:

o operant behavior, as behavior modified by its consequences, is a broad biological phenomenon critical for the adaptation and long-term survival of individuals and their species;

o tests of schedule-controlled behavior allow the reliable and quantitative examination of the effects of substances on behavior (Dews, 1972; Thompson and Boren, 1977);

o the extensive literature on operant behavior provides a conceptual framework for analysis of behavioral effects (Thompson and Boren, 1977);

o simpler procedures, such as unconditioned reflexes or motor activity, measure different aspects of behavior;

o operant conditioning allows the experimenter to tailor the behavior to the needs of the experiment (e.g., to examine the effects of a substance on a variety of response rates; Dews and Wenger, 1977, Laties, 1978);

o and tests of schedule-controlled behavior have been used to evaluate the effects of a variety of chemicals: metals (Evans, Laties, and Weiss 1975); solvents (Wood, Rees, and Laties, 1983; Glowa 1981; Colotla et al. 1979); anoxic agents (Laties and Merigan, 1979); and pesticides (Dietz and McMillan, 1979; Reiter et al. 1981).

II. RATIONALE FOR STUDY DESIGN

A. Experimental Design

Experimental designs that permit both within subject and between-groups analysis are recommended in the guideline. Each type of design has certain advantages. Within-subject comparisons provide a more accurate means of specifying individual response to treatment whereas between groups designs allow more general quantitative conclusions about the effects of a particular exposure level. Although within-subject designs are most common in studies of the effects of drugs and chemicals on operant behavior, traditional acute and subchronic toxicity studies rely almost exclusively on between-groups designs. Sidman (1960) and Kazdin (1976) contain more detailed discussion of the scientific and statistical features of within-subject studies.

B. Species

Common strains of laboratory rats or mice will generally be recommended because:

o they have been widely used in behavioral toxicology studies of schedule-controlled behavior (see e.g., volumes 1-3 of Neurobehavioral Toxicology and Teratology);

o rats are also recommended for subchronic toxicity studies (USEPA, 1982);

o data on the same species from both types of studies will facilitate comparisons of the relative potency of effects on different target organs.

For some substances, exisitng data on species sensitivity or metabolism may make it appropriate to use a non-rodent species (e.g., organophosphates, methanol). In operant conditioning studies, the most commonly used non-rodent species are pigeons and squirrel monkeys.

C. Number of Animals

The number of subjects to be exposed in each group or to each exposure level and the variability of their pre-exposure performance will be key factors in determining the magnitude of changes which can be detected. For a simple between groups design with 4 groups, EPA (1982) has calculated that, given a coefficient of variation of 0.25, with 10 animals/group, a 40% difference could be detected with 90% power at the 5% level.

Dews (1978) made similar calculations based on repeated measurements of schedule-controlled behavior in 6 mice, and with the same coeffieient of variation, 0.25, estimated that a 20% difference could be detected at the 5% level. Some of the difficulties of increasing sample size to increase test sensitivity are discussed here and in Dews and Wenger (1979).

The neuropathology guideline and the NAS (1982) recommend 6 animals/exposure level (USEPA, 1982) for neurotoxicity studies. For these reasons, (see also Dews 1972) we recommend 6-12 animals per group or exposure level. The coefficient of variation of the pre-exposure behavior should be presented.

D. Positive Controls

Many chemicals commonly viewed as stimulants (e.g. amphetamine) or depressants (e.g. barbiturates), increase or decrease response rate as a function of dose, the schedule controlling the behavior, and the rate of the pre-exposure behavior (Dews, 1972; Dews and Wenger 1977). For many test substances, there will be inadequate data to predict the direction of rate changes that may be seen and, as noted above, changes in both directions may often occur. Recommended studies should be capable of detecting response rate changes in either direction.

The choice of reference substances as positive controls should be based on the available pharmacological data on the schedule of reinforcement chosen, and the test species. In some situations, there may be such data on an analogue of the test substance. This would permit added analysis of the data on the test substance in terms of its similarity to the analogue in structure or mechanism of action.

E. Dose Levels and Dose Selection

Most chemicals administered at high enough doses will have serious behavioral effects. Yet effects at these levels may not be indicative of a graded relationship between exposure and behavior by any specific mechanism. Behavioral effects at lower exposure levels may not occur, may be related to a different or more specific mechanism of action, and may bear a complex relationship to exposure. (e.g., stimulant at low doses and depressant at high doses. See, for example, Glowa, 1981; Moser and Balster 1981; on toluene).

Dews (1972) discusses some of these and other reasons which underscore the importance of determining dose-effect relationships over a wide range of doses. He recommends and we endorse separation of doses by multiples of 3 (half log units) over a 10-30 fold range.

It may be very diffcult in some situations to establish dose levels to satisfy these criteria. There are many ways that investigators may efficiently establish appropriate dose levels. For example, Glowa et al. (1982) have used within session cumulative dosing to establish a range of effective acute doses and Tilson and Cabe (1978) advocate use of a 30 day cumulative toxicity study to establish dose levels for a 90 day subchronic study.

III. Rationale for Study Conduct

A. General

The successful conduct of any toxicity study involves the specification and control of the organism, of the exposure conditions and of the environmental variables that may affect the results. However, the most important determinant of the successful outcome of the study is the training and experience of the investigator.

To control for the confounding influence of environmental variables on a study, an investigator may: 1) systematically vary each environmental variable, observe its impact on the dependent variable, and then select a value that suits the needs of the study; 2) design the study to vary each of the environmental variables systematically between treatment groups (e.g., equal numbers of animals in each group exposed at different chamber temperature); 3) or attempt to hold conditions constant for all groups for the duration of the study.

This section will describe some of the more important factors and control procedures for operant conditioning studies (See also Dews, 1972; and Thompson and Boren, 1977). Careful description of the environmental conditions and an effort to keep these conditions constant throughout the study will be most helpful in minimizing potential confounding with exposure.

B. Chamber Assignment

The balanced assignment of animals in different treatment groups to different test chambers is intended to distribute the potential variability in behavior that may result from differences in test chambers equally across treatment groups. The conduct of all testing of each animal in the same chamber seeks to hold these potential differences constant.

Similarly, if the size of a treatment group exceeds the number of test chambers, animals should be balanced with respect to the time of day that testing occurs so that this source of variability is distributed equally across treatment groups. This is a result of concern with respect to both the time between exposure and testing and the time of day (i.e., place in the subject's light-dark cycle).

C. Duration of Testing: Post-Exposure Effects

Some chemicals may produce effects only after some delay following exposure (e.g., organophosphates, methyl mercury) or produce their most serious effects after exposure ceases (e.g., morphine). Therefore, it may be necessary to evaluate an additional group of animals following the acute or subchronic exposure regimen.

D. Schedule Selection

The objective of this requirement is to attempt to control for the influence of response rate, one of the most important and best studied determinants of drug effects on behavior (Dews and Wenger, 1977), on the effect of chemicals (see also Section C, above). Seiden and Dykstra (1977) contains a useful set of tables summarizing data from several classes of chemicals on a variety of schedules or reinforcement.

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METABOLISM

OFFICE OF TOXIC SUBSTANCES
OFFICE OF PESTICIDES AND TOXIC SUBSTANCES
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

I. PURPOSE

A. Data from studies on the absorption, distribution, excretion and metabolism of a test chemical are desirable to aid in the evaluation of test results from other toxicology studies and in the extrapolation of data from animals to man. Such studies should be done on each chemical of toxicological concern. The concern may be predicated on the level and type of toxicity observed (or anticipated) and by the magnitude of potential human exposure to the chemical. The main purpose of metabolism studies is to produce data which fortify the understanding of the safety of the chemical in consideration of its intended uses and anticipated human exposure. In addition to the general reasons stated above, a metabolism study may be performed for the following purposes:

1. To determine the amount and rate of absorption of the test chemical at different dose levels;
2. To determine the pattern of distribution of the test chemical among tissues, organs and fluid compartments at different dose levels, after single and repeated dosages;
3. To identify and, to the extent possible, quantify significant metabolites;
4. To characterize route(s) and rate(s) of excretion;
5. To determine any possible bioaccumulation (bioretention) of the test substance and/or metabolites; and
6. To determine absorption, metabolism, excretion and distribution as a function of single or repeated doses. For certain chemicals, metabolism studies may not adequately define all of these.

II. DEFINITIONS

A. Bioaccumulation (bioretention) is the uptake and, at least temporary, storage of a chemical by an exposed animal. The chemical can be retained in its original form and/or as modified by enzymatic and non-enzymatic reactions in the body.

III. TEST PROCEDURES

A. Animal selection

1. Species

The preferred species is the rat. If another mammalian species is used, the tester should provide justification/reasoning for its selection Commonly used laboratory strains should be employed. Preliminary studies may be performed in several species to develop information on comparative metabolism. Information derived from preliminary studies may help in the selection of species for subsequent toxicity tests.

2. Age

Young adult animals should be used. For specific purposes, a comparative study using very young animals may provide information about the effects of age on metabolism.

3. Sex

a. Equal numbers of animals of each sex should be used at each dose level.

b. Females should be nulliparous and non-pregnant.

4. Numbers

At least 8 animals (4 females and 4 males) should be used at each dose level.

B. Dose levels and dose selection

1. At least 2 dose levels should be used.
2. The low dose should correspond to a no-effect-level.
3. The upper dose should produce toxic or pharmacologic signs, but not severe effects or a high incidence of mortality which would prevent a meaningful evaluation.

4. The determination of absorption, tissue distribution and elimination should be studied as a function of single or repeated doses.

5. The conclusive identification of a chemical, and its metabolites, requires the use of suitable analytical methods.

C. Observation period

Animals should be kept in individual metabolism cages for 7 days after the radioactive dose or until 95 percent of the administered dose is excreted (whichever occurs first), at which time all of the animals should be killed.

D. Administration of the test substance

1. The study should be done using the oral route (capsule or gavage). If another route of administration is used, the tester should provide justification/reasoning for its selection. When vehicles are used, attention should be given to the possibility that they may interfere with the kinetics of the test chemical.

2. Labeled test material

   a. Single dose testing should be performed with an analytically pure grade of the active ingredient, usually in an isotopically labeled form.

   b. Labeled compound may not be required if sufficiently selective and sensitive physical-chemical tests for identifying the compound and its metabolites are used. The label may be radioactive such as ^{14}C, ^{35}S, and ^{36}Cl or stable such as ^{15}N and ^{18}O. In some cases, more than one label per molecule may be advantageous. Labels should be placed in positions that may be expected to follow the "core" of the molecule or significant portions thereof. If possible, one should avoid placing labels such as ^{14}C in positions from which it may be expected to enter the carbon pool of the test animal. Use of readily exchangeable labeling, should be avoided.

3. The following 4 groups of animals should be studied:

   a. Group A animals shall each receive a single intravenous dose of the labeled test substance at the low dose. If it is not possible to dissolve the test substance in physiological saline or water, this group should be omitted.

   b. Group B animals should each receive a single oral dose of the labeled test substance at the low dose.

   c. Group C animals should each receive a series of single daily oral doses of the nonlabeled test substance (by capsule or intubation) over a period of at least 14 days, followed at 24 hours after the last dose by a single oral dose (by capsule or intubation) of the labeled test substance. Each dose should be at the low dose level.

   d. Group D animals should each receive a single oral dose (by capsule or intubation) of the labeled test substance at the high dose level.

E. Observation of animals

1. Distribution

Concentration and quantity of test chemicals in the tissues and organs should be measured at the time of sacrifice.

2. Metabolism

For determining the extent of biotransformation, urine samples and fecal extracts should be analyzed by suitable techniques. Major metabolites of the chemical should be identified by appropriate methods. It is also important to determine the metabolite pattern of the test chemical after repeated dosages.

3. Excretion

When determining excretion of the test chemical by laboratory animals, the use of individual metabolism cages is recommended for collection of urine and fecal samples. The quantities of test chemical and major metabolites in urine, feces and in expired air should be measured at several time points after exposure (i.e., 4, 8, 12 and 24 hours) and daily thereafter, until approximately 95 percent of the administered dose has been excreted or until 7 days after dosing.

4. In the rat, quantities of label in urine, feces and expired air should be measured at appropriate intervals (i.e, 4, 8, 12, and 24 hours, 1.5, 2, 3, 4, 5, 6, and 7 days) throughout the study for all animals. However, if a preliminary study shows no volatile labeled materials are exhaled during the period of zero to 24 hours after dosing, such evidence may be submitted in lieu of measuring label in the expired air for this study. In the dog, quantities of label in urine and feces should be measured at appropriate intervals (i.e., every 6 hours for the first 48 hours after dosing and every 12 hours for the remaining 5 days) throughout the study for all animals.

For all animals in groups B, C, and D, the quantity of label in tissues and organs should be measured at sacrifice by suitable methods with particular attention to bone, brain, fat, gonads, heart, kidney, liver, lungs, muscle, spleen, tissues which displayed pathology (in this or prior studies), and residual carcass.

5. Urine and feces from all groups should be analyzed by suitable methods in order to determine the extent of absorption and biotransformation and to identify the metabolites. An assay method for detection of each major metabolite may be requested by the Agency.

IV. DATA AND REPORTING

A. Treatment of results

Data should be summarized in tabular form.

B. Evaluation of results

All observed results, quantitative or incidental, should be evaluated by an appropriate statistical method.

C. Test report

In addition to the reporting requirements as specified in the EPA Good Laboratory Practice Standards [Subpart J, Part 792, Chapter I of Title 40. Code of Federal Regulations] the following specific information should be reported:

1. Quantity of isotope, together with percent recovery of the administered dose, in feces, urine, and the following tissues and organs of animals in all groups: Bone, brain, fat, gonads, heart, kidney, liver, lung, blood, muscle, spleen, tissues which displayed pathology (in this or prior studies), and residual carcass;
2. Percent absorption. If possible by the oral route in groups B, C, and D.
3. A full description of the sensitivity and precision of all procedures used to produce the data; and
4. Information on the degree (i.e., specific activity for a radiolabel) and site(s) of labeling of the test substance.

5. Counting efficacy data should be made available to the Agency upon request; and

6. Species and strain.

V. ADDITIONAL METABOLISM STUDIES

Additional, more specific studies may be required to clarify important points. Some areas for possible further study include: Identification of tissue residues; binding by macromolecules in the blood, liver, gonads and other tissues; placental transfer; entrance into breast milk; biotransformation by specific organs, tissues and cell fractions; and absorption by dermal or inhalation routes of exposure. Plasma binding studies may be conducted, usually in vitro with plasma. Placental transfer of a chemical substance may be determined by dosing pregnant rodents with chemicals and assaying their fetuses for the chemical. Additional species may be utilized as the rat and dog differ significantly in metabolic pattern.